Understanding Pasture Performance - The Interaction between Perennial Ryegrass and Viruses

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Abstract

In New Zealand livestock-based agriculture is an essential component of the economy, and at the very core of the majority of livestock-based farming systems in New Zealand is a pasture comprised of perennial ryegrass (*Lolium perenne* L.). Therefore, the productivity and profitability of livestock industries is inextricably linked to, and influenced by, the quality and performance of the underlying perennial ryegrass pasture. However, previous research indicates that ryegrass pastures in New Zealand could be infected with multiple viruses, and that the incidence or "load" of viruses within ryegrass pastures could potentially increase over time. Considering the significance of ryegrass to agricultural productivity, and current targets to increase the output of ryegrass pastures, these findings are concerning, as 1) multiple virus infections could have synergistic effects, in which the impact of one pathogen could potentially be exacerbated by the concurrent presence of other pathogens within a single host, and 2) increasing viral load or incidence within a pasture could potentially undermine the productivity and persistence of a ryegrass pasture over time. However, the extent to which multiple virus infections and/or increasing viral load undermines the persistence and productivity of perennial ryegrass is yet to be ascertained. Therefore, the purpose of this research is to elucidate the potential impact of multiple virus infections and increasing viral load upon the yield and persistence of ryegrass. To determine the impact of viral load and/or multiple viruses upon ryegrass, tillers obtained from 10-year-old and 1-year-old ryegrass were screened for multiple viruses, viral load was quantified, and the yield of old and new ryegrass was compared over time**,** to ascertain if there was or is potentially a link between viral load and ryegrass performance. The results of this project indicate 1) that multiple viruses are present within the ryegrass material examined, 2) that viral load is higher in 10-year-old ryegrass, and 3) 10-year-old old ryegrass produced less biomass (4-29%) than young ryegrass. Overall, the results of this research demonstrate that there is a potential correlation or link between increasing and/or high viral load and yield deficit in old ryegrass.

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Abbreviations

BYDV	*Barley Yellow Dwarf Virus*
CYDV	*Cereal Yellow Dwarf Virus*
RGMV	*Ryegrass Mosaic Virus*
LPPV	*Lolium perenne Partitivirus*
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
CP	Coat Protein
ssRNA	Single stranded RNA
gRNA	Genomic RNA
sgRNA	Sub-genomic RNA
RdRp	RNA dependent RNA polymerase
ORF	Open Reading Frame
UTR	Untranslated Region
BTE/TE	*BYDV* independent translation enhancer element/translation enhancer
V-pg	Viral genome-linked protein
eIF4E	Eukaryotic translation initiation factor
tRNAs	Transfer RNA
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative reverse transcription PCR
ELISA	Enzyme-linked immunosorbent assay
siRNA	small interfering RNA
NTPs	Nucleoside Triphosphate
LSD	Least Significant Difference
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
OD Ratio	Optical Density
eEF1A	Eurkayotic translation elongation factors 1 alpha
TBP-1	TATA-box binding protein-1
NTC	No Template Control
PC	Plate Calibrator
GxE	Genotype x Environment Interactions
GxExV	Genotype x Environment x Virus Interactions

Chapter One

Introduction

1.1. Ryegrass - Traits of Priority:

In New Zealand, livestock-based agriculture is an essential component of the economy, comprising 69% of New Zealand's gross agricultural revenue with an estimated export value in excess of ~$20 billion (Stewart et al., 2014; NZTE, 2016). At the very core of the majority of livestock-based farming systems in New Zealand, is a pasture or pastoral system comprised primarily of perennial ryegrass (*Lolium perenne* L.) (Lee et al., 2012; Guy, 2014; Stewart et al., 2014). Perennial ryegrass is an integral part of livestock-based or pastoral farming systems, as it provides the feed necessary to sustain the range of obligate herbivores being farmed. Therefore, the productivity and profitability of New Zealand's primary or livestock-based industries is dependent upon the performance of the underlying perennial ryegrass pasture (Minneé et al., 2010; Lee et al., 2012; Guy, 2014).

Introduced in the 19th century by European settlers, perennial ryegrass, a temperate cross-pollinated grass with a high degree of self-incompatibility, became the most emergent pasture species in New Zealand (Stewart & Hayes, 2011; Lee et al., 2012; Stewart et al., 2014). The prominence of ryegrass within New Zealand's pastoral systems can potentially be attributed to the possession of a particularly valuable set of heritable traits, specifically, "production" traits (Stewart & Hayes, 2011; Lee et al., 2012). Production traits can be defined as observable phenotypic attributes that have the potential to improve animal production *in situ*, and thus they are traits that have the potential to influence and/or enhance economic gain within various farming systems (Stewart & Hayes, 2011; Lee et al., 2012). Traits that have the capacity to influence animal production are, yield or annual dry matter production, quality or the availability of metabolisable energy, and persistence or the capacity to maintain stable dry matter production over time irrespective of exposure to various forms of environmental stress (Lee et al., 2010; Stewart & Hayes, 2011; Stewart et al., 2014). The improvement of production traits has been the major objective of ryegrass breeding programs (Stewart & Hayes, 2011). However, production traits are, inherently, quantitative traits, whose expression can be modified over time by genotype by environment interactions, as such, if one wishes to achieve genetic gain or enhance production traits, such as yield, quality and persistence, all environmental factors that could potentially modify the expression of these traits must be evaluated and accounted for, including the impact of 'invisible' or unobservable pathogenic

factors that operate at the molecular level, such as viruses.

1.2. What are Viruses?

In the simplest of terms, viruses are little more than a collection of one or more nucleic acid templates, encapsidated within or by a protective protein coat (Wagner & Hewlett, 2004; Campbell & Reece, 2008; Hull, 2009). Viruses provide the ultimate example of "selfish genes". Essentially, the genetic information encoded within a viral genome has one primary objective; to ensure its own replication (Wagner &Hewlett, 2004). However, viruses lack the molecular machinery required to decipher their own genetic code (Hull, 2009). Thus, to replicate, a virus must infect a suitable host (Campbell & Reece, 2008; Hull, 2009). If a virus can successfully infect a suitable host cell, it can commandeer the host's molecular components and protein synthesizing machinery, enabling it to then translate its genome, and thus, to "replicate" the proteins and/or other components necessary for the production or self-assembly of new virions or virus particles (Campbell & Reece, 2008). New viral progeny or particles can then infect additional host cells. The intracellular spread and replication of viruses within a host can culminate in the occurrence or manifestation of observable symptoms associated with disease. However, viral infections do not necessarily lead to the production of symptoms (Wagner & Hewlett, 2004). Essentially, the outcome of viral infection and therefore, pathogenesis, is influenced by a range of factors, such as host and/or virus genotype, the concentration or titre of virus within the host, and the competence of the host's defense mechanisms (Wagner & Hewlett, 2004). Ultimately, the interaction between viruses and their host's, like the interaction between all biological organisms on Earth, is characterized by a range of complex, non-linear and dynamic processes that vary across time and space, at the biochemical, biophysical and molecular level.

1.2.1. Plant Viruses– The Global Context:

Currently, 80 genera of plant viruses are recognized, comprising over 2000 known species (Waterhouse et al., 2000; Strange & Scott, 2005; Agrios, 2005; Hull, 2009). There is scarcely a plant species that is not host to at least one virus (Waterhouse et al., 2000). However, not all plant viruses cause serious disease. Rather, pathogenesis or the development of symptoms associated with disease, is determined by the interaction(s) between viruses and their hosts (Wagner & Hewlett, 2004). Viruses are fundamentally a source of environmental stress, as such they exert pressure upon their host to evolve strategies and/or mechanisms that prolong

or ensure the host's survival (Wagner & Hewlett, 2004). As previously mentioned, host-virus interactions are dynamic and complex, as such the effects or impact of viral infection would be best described as a continuum or a range of slightly different responses, but with two appreciably distinct extremes at either end of the spectrum. Specifically, the impact of viral infection ranges from unnoticeable to devastating (Waterhouse et al., 2000; Strange & Scott, 2005). Generally, viral infections tend to be inconspicuous, but persistent, particularly in the case of perennial species (Hull, 2009). However, a lack of observable physical symptoms, does not necessarily mean that a virus is not undermining the capacity of the host to function in one way or another. Rather, viruses can undermine plant performance in subtle ways, such as decreasing their capacity to tolerate and/or withstand other environmental stress factors (Hull, 2009). As many viruses appear to be rather insidious, the damage that they actually cause or inflict upon a host may go unrecognized (Hull, 2009). Consequently, viruses could potentially be responsible for far greater agricultural losses than previously acknowledged, and with an increasing global population, that is expected to exceed 9 billion people by 2050, the United Nations Food and Agricultural Organization projects that agricultural production needs to increase by 70% to accommodate population growth (Kang et al., 2005; Hull, 2009; Zhang et al., 2009). However, achieving such an increase in agricultural output is a difficult task, particularly so, as the actual impact that viruses have upon crop production remains relatively undetermined (Rapicavoli, 2015; Trębicki et al., 2015). In the absence of robust quantifiable data regarding agricultural losses potentially attributable to viruses, it may be difficult to justify research into methods that mitigate these losses. Ultimately, if we wish to enhance agricultural output or, specifically, the production traits of any crop, such as yield or persistence, then we must appreciate the extent to which all environmental factors can potentially influence and/or reduce plant productivity *in situ* over space and time, and this includes evaluating the potential impact of plant viruses.

1.3 Plant Viruses & New Zealand Ryegrass:

Over the last century, more than 180 plant viruses have been detected in New Zealand (Pearson et al., 2006). These pathogens have the capacity to undermine the growth, production and thus, commercial value of a range of important agricultural species, such as perennial ryegrass, and previous research indicates that perennial ryegrass pastures in New Zealand could be infected with multiple viruses, and that the incidence of viruses within a ryegrass pasture may potentially increase over time (Latch, 1977; Delmiglio et al., 2010; Guy,

2014). Considering the significance of ryegrass to agricultural and current targets to increase the productivity of perennial ryegrass pastures, these findings are concerning, as multiple virus infections could have synergistic effects, in which the impact of one pathogen could potentially be exacerbated by the concurrent presence of other pathogens within a single host, and increasing viral load and/or virus incidence could potentially undermine the productivity and persistence of ryegrass pastures over time (Dairy N.Z., 2011; Guy, 2014). However, the extent to which multiple virus infections and/or increasing viral load undermines the persistence and productivity of ryegrass is yet to be ascertained.

Investigations conducted prior to this research indicate that the viruses most likely to be prevalent within ryegrass pastures throughout New Zealand are; ***Barley yellow dwarf virus*/*Cereal yellow dwarf virus*** (B/CYDV) and ***Ryegrass mosaic virus*** (RGMV) and ***Lolium perenne partitivirus*** **(**LPPV) formerly, *Ryegrass cryptic virus* (RGCV) (Webster et al., 1996; Delmiglio et al., 2010; Guy, 2014; Nibert et al., 2014; Veerakone et al., 2015). Of these 3 viruses, BYDV and RGMV are considered to be the most economically important (Coutts & Jones, 2002; Delmiglio et al., 2010).

1.3.1. ***Barley yellow dwarf viruses*** (Family: *Luteoviridae,* Genus: *Luteovirus*) are single stranded positive sense RNA plant viruses (Chomič et al., 2011; Trębicki et al., 2015). On the basis of host range, vector transmission, genome organization and sequence homology, the family *Luteoviridae* (or luteovirids) is divided into three genera; *Luteovirus*, *Polerovirus* and *Enamovirus* (Chomič et al., 2011; Ali et al., 2014; Veerakone et al., 2015). BYDV is the type species of the genus *Luteovirus*. There are currently 5 recognized serotypes or "strains" within the *Luteovirus* genus, which are differentiated on the basis of the vectors that transmit them. For example, BYDV-MAV is transmitted primarily by *Macrosiphum (Stiobion) avenae*, thus "MAV". In contrast, CYDV or *Cereal yellow dwarf virus*, formerly designated BYDV-RPV, belongs to the genus *Polerovirus* (Guy, 2014). CYDV-RPV is the only member of the *Polerovirus* genus known to infect species of *Poaceae,* and it has been reported that the incidence of BYDV MAV in ryegrass outnumbers CYDV (BYDV-RPV) 2 to 1 (Guy, 2014). Lastly, the genus, *Enamovirus*, is comprised of only one species, specifically *Pea enation mosaic virus 1*, which is not known to infect *Poaceae* (Miller et al., 2002). The luteovirids, BYDV-PAV, -MAV, and -PAS where assessed in this research, as such only these isolates will be discussed (Guy, 2014; Veerakone et al., 2015).

Host Range: BYDV has the capacity to infect over 150 species of *Poaceae*, as such, it is regarded as the most significant viral pathogen of *Poaceae* (Bisnieks et al., 2002; Henry et al., 2002; Delmiglio et al., 2010; Trębicki et al., 2015). BYDV can infect annual and perennial grasses, however, these species tend to be more "tolerant" of BYDV than cereals (i.e. they do not exhibit definitive symptoms of BYDV infection). However, annual and perennial grasses constitute a significant reservoir of viral inoculum (Delmiglio et al., 2010; Bisnieks et al., 2002).

Vector-Mediated Transmission & Movement within the Host: *Luteoviruses* are disseminated by horizontal or external transmission involving airborne-vectors, which acquire the virus from an infected plant and transmit it to a healthy plant (Astier et al., 2007). This mode of transmission facilitates long distance dispersal of the virus between potential hosts. BYDV can be transmitted by at least 25 species of aphid (Trębicki et al., 2015). However, the most prevalent BYDV vector in N.Z. is the bird cherry-oat aphid, *Rhopalosiphum padi* L, which transmits BYDV in a persistent, circulative manner. Basically, the vector ingests virus particles from an infected plant, but the virus does not replicate within the vector (Hull, 2009). Airborne or horizontal transmission is a highly effective mode of transmission, as it facilitates the continuous cycling of BYDV from perennial pastures to annual cereals, potentially increasing the incidence of BYDV viral load *in situ* (Luck & Finlay, 2011; Hull, 2014). Furthermore, it has been demonstrated that non-viruliferous aphids have a tendency to be attracted to BYDV-infected plants due to altered volatile organic compound profiles (Trębicki et al., 2015).

Circulative viruses, such as BYDV, are generally phloem-limited, thus the aphid must feed for several hours, depending on the viral concentration within the plant, to enable the virus to be acquired (Hull, 2014). Specifically, the aphid stylet penetrates the vascular bundle and phloem sieve-elements of an infected plant. The aphid acquires the virus particles as it ingests sap from the phloem (Bragard et al., 2013). Upon acquiring virus particles, the aphid becomes viruliferous, and it can then transmit the virus to healthy hosts for the duration of the it's life (Bragard et al., 2013). To infect a healthy plant, the aphid must again penetrate the vascular bundle and discharge or egest the virus particles into the phloem sieve elements of a prospective host (Hull, 2014). Once in the phloem, virions can move to a favorable replication site. In the case of BYDV, initial translation and replication occurs within the nucleus of the hosts' cell, then (-) strand RNA is transferred to the cytoplasm (Ali et al., 2014).

The intracellular movement of BYDV is mediated by a 17-kDa movement protein, which can transverse the membrane system by modifying the size exclusion limits of the plasmodesmata (Ali et al., 2014; Hull, 2014). Intracellular and systemic movement of BYDV within the host is dependent upon, 1) movement proteins altering the plasmodesmata and traversing the membrane, and 2) the capacity of the virus to overcome and/or suppress hosts defense mechanisms (Buchanan et al., 2000; Hull, 2014).

Morphology: The virions of BYDV consist of icosahedral particles 25-30nm in diameter with T=3 symmetry. The capsid is comprised of two different structural proteins (Ali et al., 2014; Hull, 2014). Specifically, the virion capsid contains 180 subunits of a major coat protein (~22kDa), and a minor 72kDa readthrough protein (Kaddachi et al., 2014). These proteins are involved in regulating external and intracellular transmission (Kaddachi et al., 2014).

Genome & Genome Organization: Luteovirids have a unicompetent/monopartite single-stranded linear messenger-sense ribonucleic acid (+ssRNA) genome, which varies in size from 5600nt (5.6kb) to 6000nt (6.0kb), depending upon the isolate (Liu et al., 2012; Ali et al., 2014). BYDV viral genomic RNA (hereafter gRNA) is comprised of 7 open reading frames (hereafter ORFs), which yield three subgenomic RNAs (hereafter sgRNA), as depicted in Figure 1.1 (Miller et al., 2002; Liu et al., 2012; Ali et al., 2014).

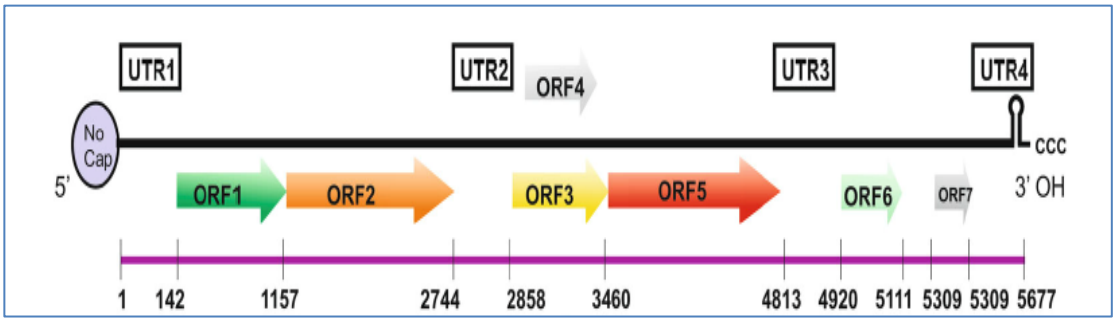


Figure 1.1. The genome of BYDV (Ali et al., 2014).

Firstly, ORF 1 encodes helicase and proteases. ORF 2, which is expressed as a frameshift from ORF1, contains RNA-dependent RNA polymerase (RdRp) (Ali et al., 2014; Hull, 2014). Helicase and RdRp are necessary for replication of viral gRNA (Hull, 2014). ORF 3 encodes the major coat protein (CP), necessary for encapsidation and, thus protection of the viral gRNA (Miller et al., 2002; Hull, 2009; Chomič et al., 2010). ORF3 also facilitates intracellular and external transmission of virions. ORF 4, which is embedded within ORF3,

encodes the movement protein (MP), which is required for systemic intracellular movement within the host (Ali et al., 2014). ORF 5 codes a minor extension of the CP and contains the readthrough domain required for aphid transmission (Miller et al., 2002; Hull, 2014). Lastly, ORF 6 and 7, a highly variable region of the *Luteovirus* genome, encodes proteins that suppress the hosts RNA silencing mechanisms (Ali et al., 2014). Although the gRNA encodes 7 ORFs, it serves as the mRNA for ORF 1 and 2 only (Ali et al., 2014). ORF 3-7 are translated from sgRNA (Miller et al., 2002).

Translation & Replication: After gaining access to the host's cells, the virion disassembles (Miller et al., 2011). Following disassembly of the virion, translation is initiated. Translation is required for viral replication and the synthesis of proteins. *Luteoviruses* replicate via the conventional (+) ssRNA system (Hull, 2014). Replication of the (+) ssRNA genome is carried out by virally encoded replicase complexes, which consists of helicase (HEL), RdRp, and host factors. The replicase complex synthesizes a complementary (-) strand, using the (+) gRNA strand as a template. The new (-) strands are then used as templates for the synthesis and accumulation of more (+) strand templates (Hull, 2009). (+) RNA strands then act as replicase templates for viral replication and the expression of encoded proteins (Hull, 2009). However, for viral replication to occur, the components of the viral replicase complex, such as RdRp and helicase, must first be translated and expressed from original viral gRNA (Hull, 2009). In BYDV, the initiation of translation is cap and poly-(A)-tail independent. Essentially, BYDV lacks a 5' cap, a 5' VPg (viral genome-linked protein) and a 3' poly-(A)-tail, components necessary to initiate translation, circularize mRNA and provide resistance to exonuclease activity (Miller et al., 2002; Ali et al., 2014). Instead, BYDV possesses a cap independent translation enhancer element (BTE/TE) in the 3' UTR (Figure 1.2) (Astier et al., 2007). The BTE initiates translation by forming a cruciform structure with three helices branching from the end of the helix, which connects the 3'BTE to the 5' terminus of the viral genome via long-distance base pairing to a complementary sequence within the 5'UTR, causing the gRNA to circularize (Miller et al., 2002; Miller et al., 2011; Ali et al., 2014; Hull, 2014). The circularization of gRNA and the cruciform BTE aids the recruitment of host 40S and 60S ribosomal subunits and host eukaryotic translation initiation factors, such as eIF4E and associated isoforms eIF3, which bind to the BTE enabling translation of ORF 1 and ORF 2 to occur (Miller et al., 2011; Ali et al., 2014).

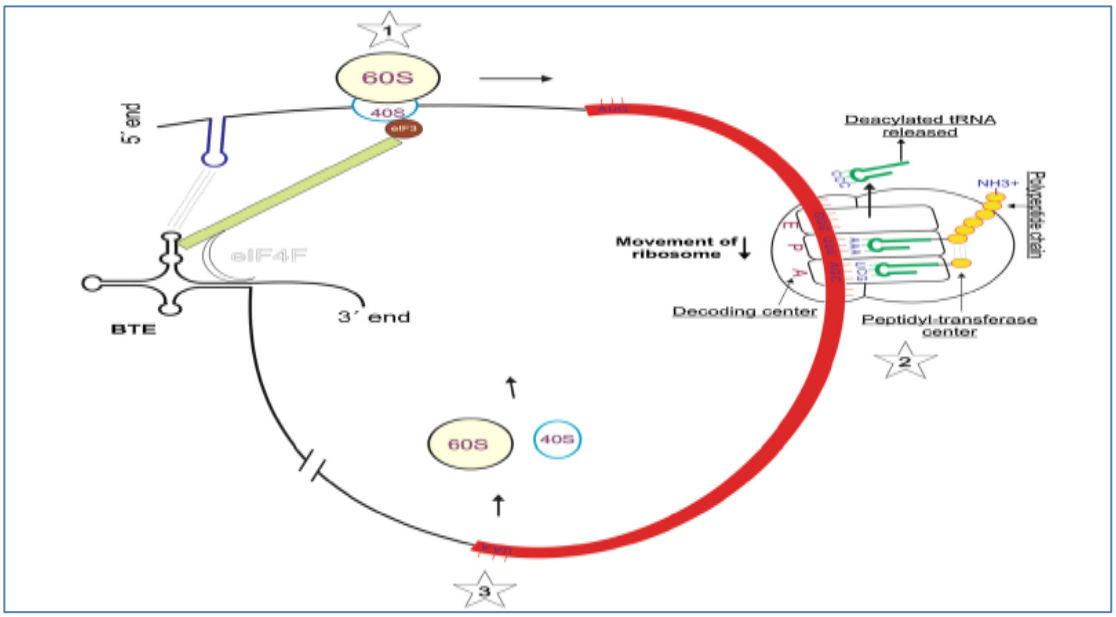


Figure 1.2. A schematic representation of translation mechanism of BYDV, demonstrating the circular formation mediated by the BTE and 5' UTR, the interaction with host factors and the binding of host-derived ribosomes and initiations factors to the BTE (Ali et al., 2014).

Translation of ORF1 and ORF2 occurs via ribosomal frameshifting, i.e. in the region of overlap between ORF1 and ORF2, ribosomes translating the ORF1 shift back one nucleotide relative to the mRNA, and resume translation in ORF2 (Miller et al., 2002; Ali et al., 2014). This ribosomal frameshifting results in the expression of BYDV or virus-specific replicase complexes, consisting of RdRp and one or more auxiliary viral replication proteins, such as helicase. RdRp accumulates causing a shift from translation to replication, in which RdRp uses the viral genome as a template for the synthesis of a complementary negative RNA strand (3'-5'). The newly synthesized strand then acts as a replicase template for the production of positive sense strands, which then accumulate and outnumber the replicase complexes (Miller et al., 2002; Ali et al., 2014). As ssRNA viruses replicate via complementary RNA (i.e. the (-) stand is used as a template for the formation of (+) strands), they go through a double-stranded (ds) RNA stage (the replicative form) (Astier et al., 2007; Hull, 2009). After the switch from translation to replication, sgRNA can then be synthesized from gRNA positive strand templates. This leads to the synthesis and expression of three 3'-coterminal mRNA's or sgRNAs required for the expression of 3' proximal genes. For example, in this case of BYDV, synthesis of sgRNA1, enables ORF4, ORF3 and ORF5 encoded products to be expressed, such as the movement proteins, the coat protein, and extension of the CP to enhance virion stability and promote aphid transmission (Ali et al., 2014; Hull, 2014). ORF6 and 7 are expressed by sgRNA2 (Ali et al., 2014).

In the early stages of infection, the (-) strand RNA is synthesized in the nucleus and then transported to the phloem for further (+) strand RNA synthesis (Ali et al., 2014). Once in the phloem parenchyma, RNA replication occurs in the sieve elements and companion cells (Ali et al., 2014). During translation and replication, host factors such as amino acids and nucleotides are used to construct viral nucleic acids and proteins (Hull, 2014). Furthermore, energy required for polymerization of viral proteins and for RNA synthesis is derived from the host, mainly in the form of nucleoside triphosphate (NTPs). Viruses also commandeer the host's ribosomes, tRNAs, associated enzymes and initiation factors (Hull, 2014). Lastly, for viral replication to be successful, the virus must overcome and suppress the hosts defense system, utilizing its own genome (Ali et al., 2014; Hull, 2014).

Infection & Symptoms: In the event that the virus can overcome the host's defense, the virus may be able to establish a full systemic infection, increasing the overall virus titre. Increased virus titre within plants can lead to the manifestation of symptoms (Hull, 2014; Kaddachi et al., 2014). BYDV induced or associated pathogenesis manifests as yellowing or reddening of the leaves, chlorosis, dwarfing, reduced root biomass, decreased rates of photosynthesis, destruction of the phloem cells, and reduced vigor (Guy, 2014; Trębicki et al., 2015). Furthermore, high virus titre is positively correlated with a reduction in yield and the possible development of more pronounced symptoms (Trębicki et al., 2015). Additionally, infected plants may also become more susceptible or less tolerant to other forms of environmental stress, such as drought. Therefore, the capacity of the infected plant to respond appropriately to the environmental stresses may be impeded. Lastly, high virus titre may reduce their plant's capacity to compete with other species, decreasing their survival and/or persistence *in situ* over time (Delmiglio et al., 2010).

Current Control of BYDV: Currently, no direct or curative countermeasures are available to control viruses, and preventative measures which target the vectors of viruses, are the only realistic form of control (Palloix & Ordon, 2011). Therefore, control of BYDV consists of the intensive prophylactic use of pesticides and chemical seed treatments (Thackray et al., 2009; Bragard et al., 2013). Essentially, control strategies do not involve "control" of the virus per se, but rather they encompass suppression of the vector population. To suppress vector populations and potential colonization of hosts, seeds are treated with imidacloprid, followed by the consistent application of insecticides, such as synthetic pyrethroids, every 3-7 weeks (Thackray et al., 2009). However, there are limitations to this form of control:

1) Reliance upon insecticides could potentially contribute to the emergence of resistance within vector populations (Thackray et al., 2009).
2) Inherently, it is difficult to accurately monitor vector populations and/or to predict epidemics, therefore, the use of insecticide may be neither "timely" nor "targeted". The use of insecticides in such an inefficient manner is environmentally irresponsible and increases the overall costs of crop production (Thackery et al., 2009; Trębicki et al., 2015). Furthermore, the use of insecticides may be entirely ineffective at minimizing and/or suppressing the occurrence of viral transmission, and thus the rates of infection (Bragard et al., 2013).
3) Lastly, targeting the vector may not necessarily reduce viral load or inoculum *in situ*, as plants and/or vectors with high virus titre may not be eradicated (Trębicki et al., 2015). Specifically, insecticides may reduce aphid colonization of plants, but it may not necessarily reduce virus transmission as viral inoculum may continue to persist within wider "untargeted" populations. Therefore, chemical control may not minimize the occurrence of future infections and/or outbreaks.

A vital component of integrated management strategies is the development and use of resistant or tolerant varieties (Bragard et al., 2013). The deployment of resistant or tolerant plants is unquestionably the best strategy for control (Bragard et al., 2013). Genetic resistance, whether host and/or pathogen-derived, is a low-cost method of control that has the potential to be highly effective, with minimal adverse environmental effects (Garcia-Arenal & McDonald, 2003; Bragard et al., 2013). However, genetic resistance often collapses, as pathogens co-evolve in response to resistance, and develop mechanisms that subvert genetic resistance. Presently, integrated management strategies to control BYDV's vectors, such as those described, are not applied to ryegrass pastures. However, extending control to ryegrass pastures, could potentially enhance the efficacy of control strategies overall (GRDC, 2013).

Distribution: In 1953, BYDV, the type species of the *Luteovirus* group, was discovered in New Zealand (Smith, 1963; Thomson & Ferguson, 1980). Since its initial detection in spring-sown wheat, BYDV has been confirmed in a range of agriculturally important crops, such as barley (*Hordeum vulgare*), maize (*Zea mays*) and ryegrass (*Lolium perenne*) (Smith, 1963; Delmiglio et al., 2010). The incidence of BYDV in ryegrass pastures throughout New Zealand was examined in 1977 (Latch, 1977), 1993 (Sinclair, 1993) and in 1996 (Guy, 2014). In 1977,

using aphid transmission tests, it was shown that the incidence of BYDV in NZ pastures varied. For example, in Palmerston North, Kaikohe and Lincoln, the occurrence of BYDV infected pastures ranged from 1-84%. In contrast, in Gore the incidence of BYDV was low 0-15% (Guy, 2014). In 1993, it was observed that in South Otago and Southland, the incidence of BYDV in ryegrass pastures was low (0-3%), but in east Otago the incidence was high 0-46% (Guy, 2014). From these surveys, it was determined that BYDV was more prevalent in some regions than in others, perhaps a consequence of vector population dynamics.

Impact: The impact of BYDV infection on perennial ryegrass is variable (Eagling et al., 1989; Clarke & Eagling, 1994). However, it has been reported that BYDV infection of ryegrass can culminate in reduced dry matter, vigor, establishment, competitiveness, quality and persistence (Clarke & Eagling, 1994; Bisnieks et al., 2002). Additionally, it has been suggested that BYDV can decrease the root dry weight of infected plants, which in turn could potentially exacerbate the impact of environmental stresses, such as drought (Catherall et al., 1987; Eagling et al., 1989; Strange and Scott, 2000; Agrios, 2005). In contrast, it has been observed that in particular genotypes or "lines" of ryegrass, BYDV produces little to no observable adverse effects (Thomson & Ferguson, 1980; Catherall & Parry, 1987; Eagling et al., 1989; Clarke & Eagling, 1994). Therefore, assessing the impact of BYDV on ryegrass is rather challenging due to the absence or poor expression of visible symptoms on particular genotypes (Eagling et al., 1989). Furthermore, yield losses attributable to BYDV are variable, indicating that yield may be a poor criterion for assessing the impact of BYDV (Catherall & Parry, 1983; Eagling et al., 1989).

Despite these challenges, attempts have been made to evaluate the potential impact of BYDV on the growth and productivity of ryegrass (Clarke & Eagling, 1994). For example, in 1980 Latch conducted field trials using pure swards of (seed-derived) ryegrass and mixed swards of ryegrass and white clover for a period of 18 months to ascertain the impact of BYDV (Latch, 1980). It was observed that after a period of 18 months the total herbage or dry matter production of the ryegrass-clover swards was not significantly affected by BYDV (Latch, 1980; Guy, 2014). However, during the study Latch observed that BYDV affected seasonal production. Specifically, in summer the virus-free swards out yielded swards in which 100% or 50% of the ryegrass was infected. Conversely, in winter the swards in which ryegrass was 50% infected, out yielded both the virus-free and 100% infected swards (Latch, 1980). Furthermore, in ryegrass-clover swards with 100% infection, clover production exceeded

ryegrass production (Latch, 1980). On the basis of his observations, Latch surmised that ryegrass-clover pastures with a high level of BYDV infection are likely to contain excessive amounts of clover during late spring and early summer, but that total herbage production would not be significantly affected (Latch, 1980). Consequently, Latch concluded that BYDV may be of "*no practical relevance to New Zealand farmers"* and although the incidence of BYDV was generally high, he stated that on well-managed ryegrass-clover pastures, BYDV may be "*of little, if any, economic importance"* (Latch, 1980). However, on the basis of Latch's observation that clover production exceeds ryegrass production in infected swards, it could be argued that this observation indicates that BYDV has the potential to reduce the vigor and competitiveness of ryegrass, which in turn could increase the likelihood that pastures will or can be invaded and successfully colonized by undesirable 'weedy' species, which could undermine pasture performance and, therefore livestock productivity (Eagling et al., 1989; Delmiglio et al., 2010).

In contrast to the impact of BYDV on clover-ryegrass swards, in the simulated swards of pure ryegrass, virus free ryegrass out yielded swards with 100% infection by **22.4%** (Latch, 1980). A similar yield deficit was observed in a separate study conducted by Wilkins & Catherall, 1977. Specifically, Wilkins & Catherall, 1977 reported that BYDV could reduce dry matter production in ryegrass-clover swards by as much as **24%** (Wilkins & Catherall 1977; Eagling et al., 1989). Wilkins & Catherall, 1977 also observed that the highest yielding genotypes tended to suffer the greater reductions in yield. On the basis of Latch's earlier observations regarding incidence rates of 84% in pastures between the ages of 6 to 15 years, and in light of estimated yield losses between 20-24% as reported by Latch, and Wilkins & Catherall, and considering that perennial ryegrass pastures in New Zealand dairy farms produce on average 14-15 t/DM/Ha/year, it follows that if a given paddock's infection rate is 84% and yield loss is potentially 22-24%, then this equates to a potential loss of ~3 t/DM/Ha/year (assuming yields of 14-15 t/DM/Ha/year) (Minneé et al., 2010). According to the Forage Value Index, this may equate to an economic loss of ~$627- $960/Ha (2016).

Furthermore, it has been observed that the effect of BYDV upon ryegrass varies depending upon the "line" or genotype and upon environmental conditions (Wilkins & Catherall, 1977; Catherall & Parry, 1987; Eagling et al., 1989). For example, in 1989 Eagling et al., assessed the impact of BYDV upon the early growth of four different commercial lines of ryegrass under two different temperature regimes. At 24°C, in all cultivars examined , root dry weight

was reduced by 30-40% (Eagling et al., 1989). Conversely, at 16°C, the effect of BYDV varied depending on the cultivar. For example, at 16°C the root and shoot dry weight of cv. Grasslands Ariki increased, but the increase was not statistically significant. In contrast, root and shoot dry weight decreased in cv. Victorian (Eagling et al., 1989). Ultimately, the findings of Wilkins & Catherall, 1977; Catherall & Parry, 1987 and Eagling et al., 1989 indicates that a GxVxE (genotype x virus x environment) interaction is present, and this tripartite interaction could potentially influence the outcome of BYDV infection. However, this interaction was not accounted for during Latch's 1980 trials, in which 150 seedlings of Grasslands Nui were evaluated under the same environmental conditions. Essentially, Latch assessed the interaction between one genotype and one virus within a single environment over a relatively short period of time (18 months) (Latch, 1980). Therefore, it could be argued that assessing the impact of BYDV upon of one genotype of perennial ryegrass within one environment may not have adequately reflected the potential impact of BYDV nor does it provide evidence necessary to substantiate the remark that BYDV may be "*of little, if any, economic importance"* in New Zealand.

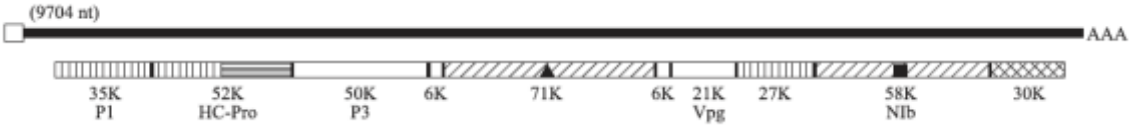
1.3.2. *Ryegrass mosaic virus* (Family: *Potyviridae*, Genus; *Rymovirus*) is the type species of the genus *Rymovirus* (Agrios, 2005). RGMV is a single stranded positive sense RNA virus, that is considered to be the most serious and widespread virus infecting temperate pasture grasses in the United Kingdom and Australia (Šutić et al., 1999; Webster et al., 1999; Raccah et al., 2001; Webster et al., 2005). On the basis of nucleotide polymorphisms within coat protein sequences, it has been determined that two distinct strains or isolates of RGMV are present in New Zealand (Webster et al., 1999). Specifically, the Kaikohe Strain (RGMV-Kaik) and the Otago Strain (RGMV-Ot) (Webster et al., 1999). RGMV-Ot is predominant in the South Island. Whilst, RGMV-Kaik has not been detected south of Marlborough (Delmiglio et al., 2010).

Host Range: RGMV has the capacity to infect a range of *Poaceae*. In addition to Italian and perennial ryegrass, RGMV can infect *Dactylis glomerata* (Cocksfoot), *Festuca pratensis* (Fescue) and *Avena sativa* (Oat) (Webster et al., 1996; Šutić et al., 1999).

Vector-Mediated Transmission & Movement within the Host: RGMV is generally, but not exclusively, transmitted by eriophyid mites, such as *Abacarus hystrix* (Nalepa) in a non-persistent manner (Webster et al., 1999; Guy, 2014). In comparison to circulative or persistent viruses, such as BYDV, which can persist or be retained within the vector for prolonged periods of time, non-persistent viruses such as RGMV only remain within the stylet of their vector for a short period of time (<24 hours) (Šutić et al., 1999; Raccah et al., 2001; Hull, 2009). Furthermore, as eriophyid mites are wind-borne they can only facilitate short-distance dispersal (Webster et al., 1996; Agrios, 2005). There are no reports to indicate that RGMV is transmitted by seed. However, RGMV can be transmitted by mechanical sap inoculation, i.e. the direct transfer of infected sap from one plant to a healthy plant (Guy, 1993; Agrios, 2005). Mechanical transmission can be facilitated by livestock treading and/or hay cutting machinery (Guy, 1993; Webster et al., 1999). In the event that a vector carrying infected sap reaches a host, and successfully penetrates the host's outer cell-wall, virions can then gain entry into the host's cells, potentially initiating infection (Astier et al., 2007). The intracellular movement of RGMV virions is not facilitated by a specific or discrete movement protein, but rather the movement of RGMV virus particles between adjacent host cells is mediated by a range of virus encoded proteins (Hull, 2009). Specifically, the coat protein, HC-pro (helper component protein) and cytoplasmic inclusion proteins operate in conjunction to facilitate movement of virus particles by increasing the permeability of plasmodesmata (Astier et al., 2007).

Morphology: The virions of RGMV are comprised of flexuous, tubular filamentous rods approximately 690-720nm in length, and 15nm in diameter with helical symmetry. Essentially, coat protein subunits are arranged in a helical manner on the external surface of the virus particle (Webster et al., 1996; Agrios, 2005; Astier, 2007).

Genome & Genome Organization: RGMV has a single stranded monopartite linear messenger (+) sense RNA genome approximately 9.7kb (Agrios, 2005; Hull, 2009). The positive sense strand of RNA encodes only one ORF, which is translated into one polyprotein that is subsequently cleaved by virally encoded proteinases, yielding 9 discrete proteins, as depicted in Figure 1.3.



5' Vpg-	P1 35K	HC 52K	P3 50K	6K1	CI 71K	6K2 21K	NIa	NIb	CP30K	- (AAA) '3

Translation

P1	HC	P3	6K1	CI 72k	6K2	NIa	NIb	CP

P1	HC	P3	6K1	CI	6K2	NIa	NIb	CP

Post- translation.

Figure 1.3. The genome organization of RGMV, followed by translation and post-translational processes. Abbreviations: VPg, viral genome-linked protein; P1, P1 proteinase, HC, helper-component proteinase; P3, P3 protein; 6K1, 6K1 protein; CI, Cytoplasmic/cylindrical inclusion protein; 6K2, 6K protein; NIa, nuclear inclusion protein; NIb, nuclear inclusion protein; CP, coat protein (Nettleship & Foster, 2000; Hull, 2009).

Essentially, the genome is translated as one large polyprotein. During post-translation the polyprotein is cleaved by viral encoded proteinases, producing 9 distinct viral proteins with discrete and overlapping functions. Potyviral proteins were assigned the following functions on the basis of comparing nucleotide sequences of known functions, and via the study of engineered mutants (Nettleship & Foster, 2000). In order from the N-terminus (5'-3'), the protein designated P1 (35K) is a serine proteinase, which cleaves the polyprotein, and also facilitates RNA binding and cell-cell movement (Nettleship & Foster, 2000; Hull, 2009). HC-Pro or HC (52K) is a cysteine proteinase, which cleaves the polyprotein, but also facilitates insect transmission, replication, cell-cell movement and suppression of host defense mechanisms (Nettleship & Foster, 2000; Agrios, 2005; Hull, 2009). The P3 (50K) protein regulates host range and pathogenicity, potentially in conjunction with the 6K1 protein, whose specific function remains unknown (Nettleship & Foster, 2000; Agrios, 2005; Hull, 2009). The proteins of CI (71K) mediate cell-to-cell movement, and RNA replication (Nettleship & Foster, 2000).

The CI (71K) and 6K proteins also facilitate the formation of intracellular aggregates known as cytoplasmic and/or cylindrical inclusions (Nettleship & Foster, 2000; Agrios, 2005). 6K2 (21K) mediates membrane anchoring during replication, and VPg replication, by interacting with eukaryotic translation initiation factor (eIF4E) and associated isoforms (Nettleship & Foster, 2000; Hull, 2009). 6K2 (21K) also encodes a cysteine proteinase, and is involved in priming RNA synthesis (Nettleship & Foster, 2000). NIa encodes proteinase, but also facilitates translation of genomic RNA and the development of nuclear inclusions. NIb also promotes nuclear inclusions, whilst encoding RNA dependent RNA polymerase (RdRp) necessary for replication of viral genomic RNA (Nettleship & Foster, 2000)**.** Potyviruses such as RGMV, form aggregates in the nuclei of infected cells, and NIa and NIb proteins, facilitate this process (Nettleship & Foster, 2000). Lastly, the CP 30K protein promotes encapsidation, insect transmission, cell-cell movement, replication, and pathogenesis (Nettleship & Foster, 2000; Agrios, 2005).

Translation & Replication: RGMV utilizes the same replication mechanism as BYDV, specifically, it replicates via the conventional (+) ssRNA system (Hull, 2014). Replication of the (+) ssRNA genome is carried out by virally encoded replicase complexes (RdRp), which consists of helicase (HEL), RdRp, and host translation initiation factors. The replicase complexes synthesize a complementary (-) strand, using the (+) gRNA strand as a template. The newly processes (-) strands are then used as templates for the synthesis and accumulation of (+) strands (Hull, 2009). (+) RNA strands then act as replicase templates for viral replication and the expression of encoded proteins (Hull, 2009). However, RGMV differs from BYDV in that it's genome possesses a VPg (virus-linked genome protein) that is covalently linked to the 5' terminus and a 3' polyadenylated tail (Agrios, 2005; Hull, 2009). The VPg and poly-(A)-tail regulate the initiation of translation (Astier, 2007; Miller et al., 2011; Nag & Pogany, 2011). Specifically, the 5' VPg and 3' poly-(A)-tail act in conjunction with each other, and host-derived factors, such as eukaryotic initiation factors (which bind to the VPg), to stimulate translation of viral gRNA (Astier et al., 2007; Miller et al., 2011; Nag & Pogany, 2011). In comparison to BYDV, the translation of RGMV is dependent upon 5' and 3' translation elements. Furthermore, RGMV's genome is initially expressed a polyprotein, which is then autoproteolytically cleaved by viral encoded proteinases, as previously stated (Hull, 2009).

Infection & Symptoms: The manifestation of symptoms associated with RGMV infection is influence by a range a factors. Essentially, genotypic differences between hosts and between viral strains can affect the overall outcome of infection (A'Brook & Heard, 1975; Wilkins & Hide, 1976; Eagling et al., 1992; Webster et al., 1999). For example, RGMV infection of tolerant genotypes may not result in the development of observable pathogenesis, such as mosaic necrosis, but these genotypes may experience a loss in yield (Wilkins, 1976; Catherall, 1987; Webster et al., 1996). In contrast, RGMV infection of sensitive genotypes may result in both significant yield losses and the development of visible symptoms, such as mosaic necrotic or chlorotic lesions (Wilkins, 1976; Webster et al., 1996). The pathogenicity of the strain will also influence the impact and/or the outcome of infection, i.e. a mild strain vs a virulent strain. Furthermore, environmental factors may also influence the impact of viral infection. For example, following the application of nitrogen, the impacts of RGMV upon ryegrass, reportedly increased in severity (Eagling et al., 1992; Webster et al., 2005). Additionally, potyviruses such as RGMV, can induce the formation of distinctive cytoplasmic and/or nuclear inclusions which are aggregate formations comprised of virus particles (Raccah et al., 2001; Astier et al., 2007). Cytoplasmic inclusions can occur in two forms, pinwheels or cone-shaped cylindrical structures (Hull, 2009), as depicted in Figure 1.4.

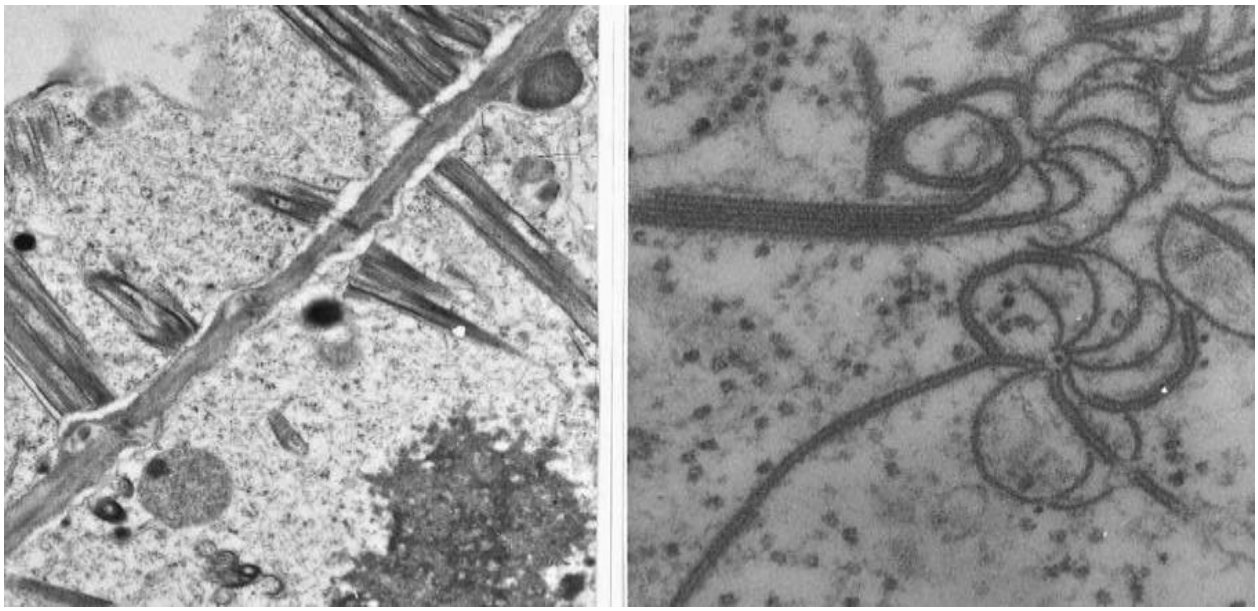


Figure 1.4. Cylindrical or cone-shaped cytoplasmic inclusions (right), and pinwheel inclusions (left) (Agrios, 2005).

Current Control of RGMV: Like BYDV, there are no direct or curative countermeasures available to control plant viruses, such as RGMV. The only widely available/accessible form of control involves the large-scale application of insecticides. In the case of RGMV, this

would involve the prophylactic use of acaricides (Guy, 1993; Webster et al., 1996). However, this approach is subject to the same limitations as BYDV, and essentially, the large scale application of pesticides is unpractical within the pastoral setting. Furthermore, as the incidence or load of BYDV, and RGMV may increase over time within a pasture, it be may appropriate in some circumstances to renew pastures at 4-5 year intervals (Webster et al., 1996). However, considering the rate at which new ryegrass can potentially become infected, and considering the rate at which viral load increases, pasture renewal may only provide short-term control (<1-4 years). Ultimately, as with BYDV, the most effective control strategy would involve the development of resistant, or preferably, tolerant varieties of ryegrass, as opposed to the large scale use of insecticides. Presently, within pastoral systems, RGMV and/or the vector of RGMV is not subject to any form of control.

Distribution: RGMV was initially detected in New Zealand in 1992 (Guy, 2014). Since its initial detection in annual ryegrass (*Lolium multiflorum* Lam) in Dunedin, RGMV and its predominant vector, *A. hystrix,* have been detected in perennial ryegrass pastures throughout New Zealand (Webster et al., 1996). In 1996, using indirect-ELISA, RGMV was detected in 7 mature (>4 years old) North Island pastures and in 60% of 20 mature South Island pastures (Webster et al. 1996). The incidence of RGMV in North Island pastures ranged from 6-60%, whereas in South Island pastures incidence ranged from 0-34% (Webster et al., 1996). In pastures used for hay, RGMV incidence was reportedly 2-24%, conversely, in irrigated pastures incidence is 4-34% (Guy, 2014). These results indicate that RGMV tends to be more prevalent in mature (>4 years) irrigated pastures (Guy, 2014). However, it has been proposed that greater incidences of RGMV within mature pastures may be attributable to the prevalence of eriophyid mites, as opposed to pasture age (Guy, 2014). Essentially, the abundance of eriophyid mites within a pasture could potentially influence the transmission and therefore the incidence of RGMV within a pasture (Webster et al., 1996). Like BYDV, the incidence and/or distribution of RGMV is potentially a consequence of vector population dynamics.

Impact: RGMV can have serious effects upon the yield and persistence of ryegrass pastures (Guy, 1993). For example, it has been reported that RGMV can reduce dry matter production by as much as 50%, and losses in dry matter production can be associated with a concomitant increase in the prevalence of "weedy" species within pastures (A'Brook & Heard, 1975; Wilkins & Hide, 1976; Eagling et al., 1992; Webster et al., 1999). Furthermore, it has been reported that RGMV can significantly reduce carbohydrate content, digestibility, vigor and

persistence (Eagling et al., 1992; Webster et al., 1996; Šutić et al., 1999; Webster et al., 2005). Traditionally, the presence of chlorotic streaks and mosaic flecks upon ryegrass provided an initial indication of RGMV infection (Webster et al., 2005). However, the impact of RGMV can vary depending upon a range of factors, such as viral strain, host genotype and environmental conditions (Webster et al., 1999). For example, in 1992, Eagling et al., observed that in Australia cultivars Ellett and Victorian infected with a Victorian isolate of RGMV, visible symptoms manifested as mosaic flecks. In contrast, in other lines of ryegrass, RGMV infection results in little to no observable symptoms (Catherall, 1987; Guy, 1993; A'Brook & Heard, 1975). For example, from ryegrass collected from three N.Z. pastures, it was found that less than half of the plants infected with RGMV exhibited visible symptoms (Webster et al., 2005). These finding demonstrate that a GxV interaction may potentially be present. Additionally, the impact of RGMV varies depending upon environmental conditions or/and pasture management practices (Gibson & Heard, 1979; Catherall & Parry, 1983; Eagling et al., 1989; Webster et al., 2005). For example, adverse environmental conditions may influence the severity of infection by exacerbating physiological stress, and seasonal variation may alter the incidence and distribution of vectors (A'Brook & Heard, 1975; Webster et al., 1996). These findings indicate that a GxVxE interaction may be present. Although assessing the impact of RGMV will be challenging, one could argue that in light of its widespread incidence (0-60%) in NZ pastures >4 years, and estimated DM production losses in the range of 10-50%, RGMV may be a virus of considerable economic importance.

1.3.3. *Lolium perenne Partitivirus* (LPPV): Formerly *Ryegrass Cryptic Virus*, (Family *Partitiviridae*, Genus Alphacryptovirus), but recently designated *Lolium perenne Partitivirus* (Family *Partitiviridae*, genus *Deltapartitivirus*) on the basis of phylogenetic analysis (Nibert et al., 2014). LPPV is a double stranded RNA virus, that was initially detected in Italian Ryegrass *(Lolium multiflorum*) by R. Plumb in 1973 (Šutić et al., 1999). It has since been detected in New Zealand cultivars of Italian ryegrass (*Lolium multiflorum*), specifically cv. Tama and in hybrid ryegrass (*L. multiflorum x perenne)* cvv. Ariki and Manawa (Nibert et al., 2014; Guy, 2014). Whilst the presence of LPPV has been confirmed, the incidence and/or distribution of this virus within New Zealand ryegrass pastures has not yet been evaluated or documented in any of the literature reviewed to date.

Morphology: Partitiviruses, such as LPPV, are comprised of biparticulate (two) isometric virions approximately 29-30nm in diameter, with T=2 symmetry, and coat proteins arranged in an T=1 icosahedral lattice, as depicted in Figure 1.5 (Hull, 2014; Nibert et al., 2014).

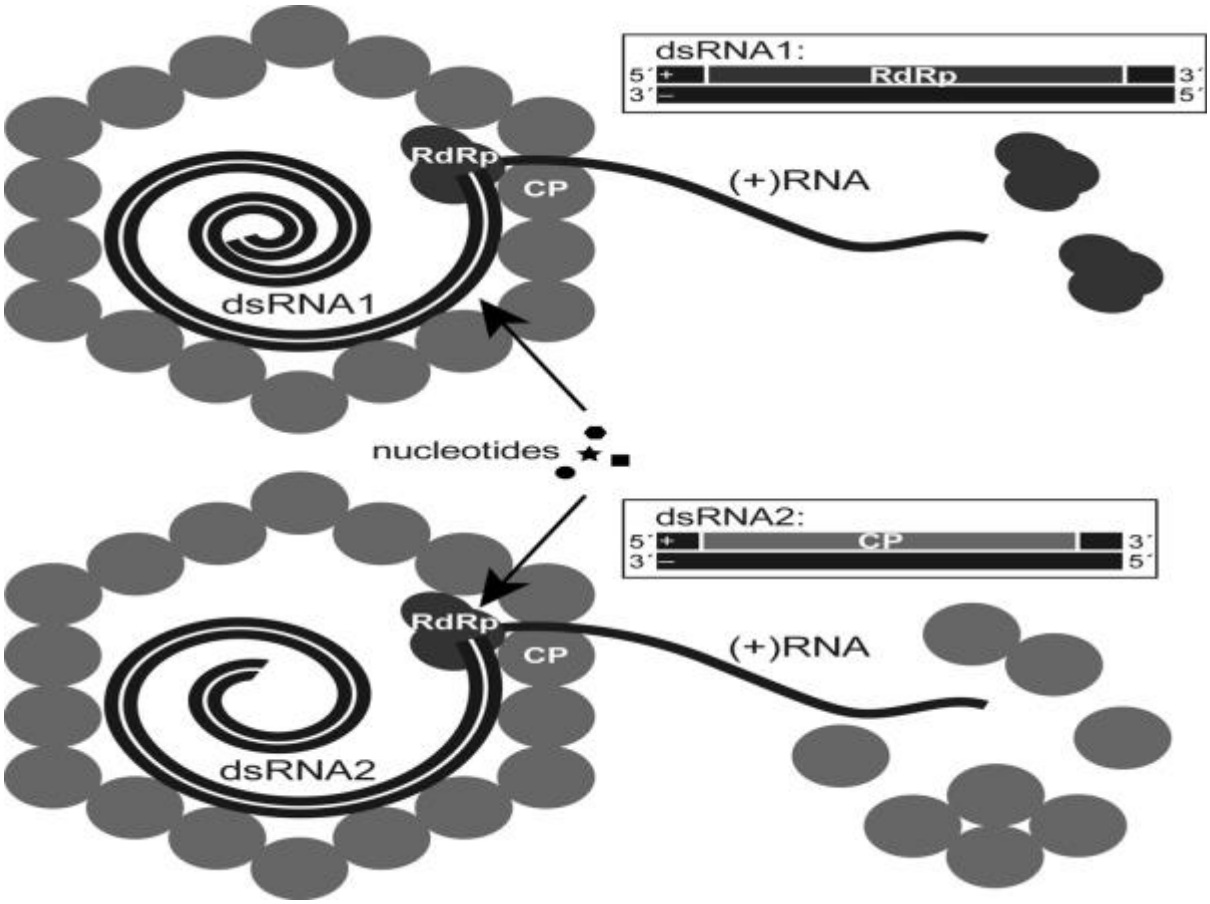


Figure 1.5. A depiction of *partitivirus* particles and genome segments (Nibert et al., 2014).

Each virion or particle contains RdRp molecules, as depicted above, that are potentially non-covalently attached to the inner capsid surface (Hull, 2014; Nibert et al., 2014).

Genome & Genome Organization: LPPV possess a monopartite but bisegmented dsRNA genome, each of which is individually encapsidated (Nibert et al., 2014). Each virion contains one dsRNA genome, and each dsRNA genome contains a single ORF (Hull, 2014). One dsRNA segment is larger than the other (Hull, 2014; Nibert et al., 2014). Specifically, the larger 2.0kbp, 73kDa genome, encodes RNA dependent RNA polymerase (RdRp) necessary for replication, and is designated dsRNA1 (Hull, 2014; Nibert et al., 2014). The smaller 1.5kbp, 54kDa segment, encodes the coat protein (Lesker et al., 2011; Nibert et al., 2014). Each genome segment contains conserved sequences in the 3' UTR (20nt in dsRNA1, and 10nt in dsRNA2) and 3' poly(A)-tail (Lesker et al., 2011). Partitiviruses, such as LPPV do not

appear to encode movement proteins or proteins that facilitate external or vector-based transmission (Liu et al., 2012; Nibert et al., 2014).

Extracellular and Intracellular Transmission: LPPV is vertically transmitted, or specifically, it is seed-borne (Guy, 2014). Other modes of transmission remain unknown (Šutić et al., 1999; Hull, 2009). Partitiviruses such as LPPV do not encode proteins that mediate horizontal transmission, or facilitate intracellular movement within the host (Liu et al., 20112; Nibert et al., 2014). Rather, LPPV is externally transmitted via gametes (pollen or seeds), and intracellularly via cell division (Roossinck, 2010; Liu et al., 2012; Nibert et al., 2014)

Translation & Replication: For LPPV to successfully infect and replicate within a host, two virions or particles must be present within a host cell (Nibert et al., 2014). If this occurs, then replication is possible. Whilst LPPV possesses a bisegmented dsRNA genome, the process of translation and replications is analogous to that of ssRNA viruses. Essentially, RdRp molecules packaged within the virions of LPPV, and associated host-derived factors such as nucleotides, facilitate the production of a complementary (+) sense RNA stranded on the basis of the (-) negative sense strand of the dsRNA genome (Dickinson, 2003; Nagy & Pogany, 2011; Nibert et al., 2014). Partitiviruses replicate via a semi-conservative mechanism (Dickinson, 2003; Nibert et al., 2014). Basically, once a newly synthesized (+) strand has been produced, it is retained within the virion as part of the dsRNA genome, whilst the original (+) strand is released or extruded from the virus particle to serve as a template for translation, as depicted in Figure 1.5 (Dickinson, 2003; Nagy & Pogany, 2011; Nibert et al., 2014).

Infection & Symptoms: Partitiviruses, such as LPPV, rarely have deleterious effects upon their hosts, and new research suggests that a mutualistic relationship may develop or exist or between persistent Partitiviruses and their hosts (Roossnick, 2010; Guy, 2014; Nibert et al., 2014). Firstly, it has been proposed that persistent viruses such as LPPV, may provide cross-protection or confer resistance to more acute or chronic plant viruses (Roossnick et al., 2010; Nibert et al., 2014). At this point it must be mentioned, that the definition of "persistent" in relation to LPPV, differs from persistent in terms of vector transmission (Roossnick et al., 2010). Specifically, Partitiviruses are described as persistent, as their mode of action or "lifestyle" differs from that of acute or chronic viruses, such as RGMV and BYDV

(Roossnick et al., 2010; Liu et al., 2012). A virus is defined as persistent, when it is vertically transmitted, but does not cause observable or obvious symptoms to manifest, whilst maintaining long term infection of a host that is characterized by consistently low titres (Roossnick, 2010).

Recent findings indicate that persistent cryptic viruses may have positive effects on their hosts (Nibert et al., 2014). For example, when a cDNA library of white clover (*T. repens*) was screened, it was observed that the sequence encoding the *T. repens* gene, *TrEnodDR1* (*Trifolium repens early nodulin downregulation* 1*)*, which regulates root nodulation formation by *T. repens*, was analogous to the CP-encoding dsRNA2 segment of White Clover Cryptic Virus-1. It was proposed that the CP gene of WCCV-1 could potentially be exploited by *T. repens* to facilitate root-nodulation (Nibert et al., 2014). When a similar study was conducted with regards to cDNA clones derived from *Lolium perenne*, a sequence analogous to the RdRp of a Deltapartitivirus was identified (Nibert et al., 2014). On the basis of this finding, LPPV was assigned to the genus *Deltapartitivirus* (Nibert et al., 2014).

However, despite these findings, it is plausible that LPPV could have a negative impact upon it's host. For example, it has been observed that *Beet cryptic virus* decreases yield, and when ryegrass is infected with RGMV and LPPV, the symptoms of RGMV are exacerbated (Eagling et al., 1992; Guy, 2014). Thus, it is likely that LPPV and RGMV act synergistically, enhancing the negative effects of RGMV, as opposed to LPPV providing cross-protection or conferring resistance to RGMV (Guy, 2014). It could be hypothesized that LPPV enhances the impact RGMV, potentially as a result of viral encoded RNAi suppressor proteins, which subdue the host's RNAi silencing mechanisms (Waterhouse et al., 2001; Obbard et al., 2009; Alvarado & Scholthof, 2009). Essentially, the suppression of host RNAi defense mechanisms by one virus could facilitate the replication of another virus present within the host. It is equally possible that the concomitant presence of multiple pathogens could overwhelm a plants defense mechanisms, increasing the likelihood that the host's defenses could be overcome by one or both pathogens (Hull, 2009). Understanding the interaction between RGMV, LPPV and other viruses known to infect ryegrass may be valuable, particularly in light of recent findings which indicate that ryegrass pastures in New Zealand can be infected with multiple viruses (Delmiglio et al., 2010; Guy, 2014).

Host Range: To date, LPPV has only been confirmed in species of perennial, Italian (*Lolium multiflorum)* and hybrid (*L. multiflorum x perenne)* ryegrass in New Zealand (Delmiglio et al., 2010; Liu et al., 2012; Guy, 2014). The capacity of LPPV to infect other species remains unknown.

Current Control: The transmission and/or incidence of LPPV is not controlled within ryegrass pastures, as this virus is not generally considered as problematic.

1.3.4 Multiple Virus Infections & Ryegrass

The observation that ryegrass pastures may be infected with multiple viruses is concerning, as although the impact of a single virus infection may potentially be negligible, multiple virus infections may actually have considerable consequences in terms of productivity and persistence over time, as multiple virus infections can or may act synergistically, in which the impact of one virus could potentially be exacerbated by the concurrent presence of other viruses within a single host, as has been observed with LPPV and RGMV (Eagling et al., 1992; Guy, 2014). Specifically, when ryegrass is infected with RGMV and LPPV, the symptoms of RGMV become more severe, Thus, it is likely that LPPV and RGMV act synergistically (Clarke & Eagling, 1994; Guy, 2014). However, a synergistic interaction may not exist between all viruses. For example, in 1987, Catherall assessed the combined effects of BYDV and RGMV upon perennial ryegrass. When both viruses where present within the same host, the extent of the damage was comparable to the damage caused by the "*most damaging virus on its own"* (Catherall, 1987). However, the extent to which multiple virus infections undermine the persistence and productivity of ryegrass in NZ remains unclear.

1.4 Other Implications of Plant Viruses

In New Zealand, is it common for pastures to be maintained and utilized for 18-30 years (Stewart et al., 2014). However, this could potentially be problematic, as in 1977 it was observed that the incidence of BYDV in ryegrass pastures increased over time, and that the highest rate of virus infection occurred in pastures aged 8 to 15 years (84%) (Latch, 1977). Additionally, in 1996, it was observed that RGMV tended to be more prevalent in mature pastures (Webster et al.,1996). Although, the higher prevalence of RGMV and/or BYDV in mature pastures may be the result of vectors, as opposed to pasture age, it remains, that higher incidences of RGMV and BYDV have been observed in older pastures than in younger ones. Additionally, in 1989, it was suggested that the impact of viruses, such as BYDV could

potentially become more apparent or severe with the age (Eagling et al., 1989). In the event that viral load and/or incidence does indeed increase with time or the severity of viral infection increases with time, then this could potentially undermine pasture persistence.

Pasture persistence can be defined as stable yield production and/or performance of a desirable species through time without major intervention (Stewart et al., 2014). Persistence is generally quantified or measured as dry matter production and/or the proportion or density of a desirable species within a pasture over time (Cosgrove, 2011; Stewart & Hayes, 2011; Stewart et al., 2014; Tozer et al., 2011). If a decrease in dry matter production is observed or if a desirable species is increasingly replaced by "weedy" species, then this constitutes a decline in persistence (Stewart et al., 2014; Tozer et al., 2011). Increasing pasture persistence has become a primary breeding objective in New Zealand, and since the late 1990's researchers have focused on determining which factors influence and/or reduce persistence, and minimizing their impacts (Brazendale et al., 2011; Stewart et al., 2014). On the basis of farmer's observations and pasture research, physical or abiotic environmental stresses and grazing/pasture management practices are considered to be the most important factors influencing pasture persistence (Tozer et al., 2011). Whilst viruses and increasing viral load have not been considered as factors that could potentially influence or undermine ryegrass persistence, despite the findings of previous research, as stipulated above. Thus, the impact that viruses have upon ryegrass over time, or specifically, the impact that viruses have upon ryegrass's ability to maintain stable dry matter production and "presence/abundance" within a pasture remains unknown.

Vector-transmitted viruses such as BYDV and RGMV could have other implications for N.Z. agriculture that have not yet been considered. For example, the fourth assessment report released by the IPCC in 2013 predicts that global temperatures will increase between 0.4°C and 5.4°C by 2080 (Trębicki et al., 2015). Researchers have postulated that elevated temperatures may lead to a concomitant increase in aphid persistence, abundance and dispersal, such as *Rhopalosiphum padi* L. (Hempitera; Aphididae), the bird cherry-oat aphid (Finlay & Luck, 2011; Nancarrow et al., 2014; Trębicki et al., 2015). In New Zealand, at least six BYDV aphid vectors are present, but *R. padi* is the most prevalent (Teulon et al., 2008). *Rhopalosiphum padi* transmits all major strains of BYDV, either alone or in combination, in a persistent, circulative manner (Finlay & Luck, 2011; Nancarrow et al., 2014). Once infected, *R. padi* can remain infective for life (Finlay & Luck, 2011). As increasing global temperatures

are likely to have an impact on aphids, it is equally likely that elevated temperatures will have implications for BYDV epidemiology (Nancarrow et al., 2014). For example, increased abundance, migration and greater fecundity of aphids under elevated temperatures, could potentially facilitate the continuous cycling of BYDV from perennial pastures to cereal annuals and native grasses, potentially increasing the rate of transmission and incidence of BYDV viral load *in situ* (Finlay & Luck, 2011). Increasing temperature could potentially alter the persistence and/or abundance of a range of insects, and possibly increase the transmission of a range of viruses, including RGMV.

Additionally, an increase in vectors and viral transmission could potentially modify the evolutionary potential of insect-transmitted viruses such as BYDV and RGMV (Jones, 2009; Finlay & Luck, 2011). Viruses, and in particular, (+) sense ssRNA viruses such as BYDV and RGMV, are capable of producing novel strains and genetic variants rather rapidly (Roossinck, 1997; Garcia-Arenal & McDonald, 2003; Wu et al., 2011). Genetic variation within the viral genome can be generated with each replication event, as the rate of mutation is several orders of magnitude higher than eukaryotes (Astier et al., 2007; Wu et al., 2011). For example, in strains of BYDV the genome evolves at an average rate of 3.158×10^{-4} (Wu et al., 2011). However, mutations or new genetic variation within the genome must be beneficial, rather than deleterious (Elena et al., 2014). Viral evolution is aided by increasing: 1) population size, 2) the connectivity between hosts and pathogens (gene flow), and 3) the rate of viral replication events (Garcia-Arenal & McDonald, 2003; Elena et al., 2014; Hull, 2014). Increasing the transmission of any vector-transmitted virus could increase the evolutionary potential of the wider consensus population. Thus, epidemics could become more likely as the rate at which novel strains evolve increases.

1.5. Research Objective:

Previous research suggests that 1) the incidence or "load" of virus within a given pasture could potentially increase with time, 2) that the severity of viral infection could increase with age, 3) that pastures may be infected with multiple viruses, and 4) that viruses such as BYDV and RGMV, which are known to decrease ryegrass yield and persistence, and have been detected within New Zealand ryegrass pastures (Guy, 2014). However, despite these findings, viruses have not and are not traditionally considered as an issue to the pastoral or agricultural sector, and previous research tends to reflect this bias (Cosgrove et al., 2011). Specifically,

previous research has focused on the impacts of single virus infections without attempting to quantify or ascertain if viral load increases with time, if multiple viruses are present or if the severity of virus infection is exacerbated with age. Additionally, previous attempts to determine the impact of a single virus upon perennial ryegrass have not adequately demonstrated the actual or true extent to which virus infections can affect New Zealand ryegrass, as previous researchers have not accounted for genotype x environment or genotype x environment x virus interactions. Therefore, the actual impact that viruses could have upon ryegrass remains unclear. Considering the importance of perennial ryegrass to New Zealand's agriculture, it is necessary to elucidate that potential impact of increasing viral load and/or multiple viruses upon ryegrass. Therefore, the primary purpose of this research is to determine the impact of multiple viruses and the impact of viral load upon the yield and persistence of perennial ryegrass. Specifically, ryegrass will be screened for multiple viruses and viral load will be quantified to determine if an increase in the concentration of viruses within ryegrass has a negative impact upon yield and persistence.

Currently, the actual impact of viruses, and the link between yield losses and pasture persistence and viruses remains unclear. This research could facilitate greater understanding of the impact that viruses have upon ryegrass yield and persistence, and enable secondary impacts or outcomes of viral infection to be identified, such as decreased competitiveness and/or increased susceptibility to other forms of environmental stresses. Furthermore, increasing our understanding of the impact of viruses on ryegrass could be beneficial as pasture management practices could be refined or new-strategies implemented to reduce or minimize agricultural losses potentially attributable to viruses. However, in the absence of a clear understanding of how viruses impact ryegrasses' performance, the status quo is likely to maintained, despite potential agricultural losses that could be occurring. In light of what has been discussed, **it can be hypothesized that increased viral load and/or multiple virus infections could potentially reduce the yield and persistence of perennial ryegrass overtime**. In order to assess the potential implications of viral load and/or multiple virus infection on ryegrass yield, or in order to prove or disprove the hypothesis, the following research objectives and approaches were developed.

Chapter Two

Materials and Methods

Technical Objective #1 – Comparative analysis of ryegrass yield and persistence:

Whilst one may intuitively expect that the productivity of new ryegrass should exceed that of old ryegrass, at the outset of this research there was no quantitative data to support such an assertion. Subsequently, it was necessary to conduct our own comparative analysis of old and new ryegrass over time to obtain quantitative data in order to satisfy the first technical objective of this research. Specifically, to determine if the yield and/or persistence of old and new ryegrass actually differed. To quantify and compare pasture yield and persistence during the course of this research, a field trial of comparator plots or swards of ryegrass from 10-year-old (tiller derived ryegrass) and 1-year-old or 'new' (seed derived ryegrass) was developed. The dry weight or dry matter of each comparator ryegrass plot or sward was obtained, measured and compared overtime to enable us to determine, in a quantitative manner, if the performance of old and new ryegrass actually differed. Furthermore, to ensure that GxVxE interactions were accounted for 5 different lines of ryegrass were compared. These 5 lines were subjected to the same treatment and environmental variation over time (10 months). Once yield data was obtained from the 10-year old and 1-year-old or new ryegrass plots for each of the five lines, it was subjected to statistical analysis using GenStat 16 software (VSN International, Ltd.) and Minitab version 17 (Minitab® Statistical Software, Inc, USA). If the performance or dry matter production of old and new differed, it was necessary to then determine if differences in performance, specifically, yield and persistence, were linked to differences in viral load and/or multiple virus. To facilitate both a comparative analysis of between old and new the following approaches were used.

2.1 Biological Material:

To obtain biological material for use during the course of this research, ryegrass tillers, without endophyte, ~10-years-old were taken at random from fairy ring structures. Fairy-ring structures, such as that depicted in Figure 2.1, are comprised of clonal material, as such, the tillers obtained from these structure should possess the same genotype, and should be of equal age (~10-years) and physiology.

Figure 2.1. Fairy-ring grass structures at paddock R6, Agriseeds.

For each line of ryegrass assessed during this research, 24 individual fairy-ring structures were selected at random and from each of the 24 fairy ring structures, ~20 tillers were removed to obtain a total of ~480 tillers per line. From ~480 tillers, 60 tillers were selected at random and planted at equal densities into 6 different (37.5cm x 23cm) trays as depicted in Figure 2.2, to develop 6 replicates of old plant material representative of that particular line of ryegrass. Once developed, the trays comprised of old ryegrass material were placed in a polytunnel and watered until established.

Figure 2.2. Ryegrass swards comprised of ~10-year-old tillers obtained from fairy-ring structures at Agriseeds.

To develop comparator swards of new material, the original seed used to establish the ~10-year-old ryegrass plots was obtained from Agriseed's germplasm collection. For each of the corresponding lines, 60 seeds from a batch of 360 were selected at random and planted into 6 trays at equal densities and distribution to the corresponding old tillers. To achieve standardization across each replicated minisward for old and new, the same tool was used as depicted in Figure 2.3.

Figure 2.3. Standardization of comparator swards.

To ensure that standardization between tiller derived and seed derived swards was maintained, germination counts were conducted. If seeds within the swards failed to germinate, they were removed and replaced with spare seedlings.

To account for genotypic variation, and potential GxVxE interactions, 5 different cultivars or lines of ryegrass were used during this research, as listed in Table 2.1. Specifically, tillers and seeds from 5 different cultivars were used to establish comparator swards. Thus, for each of the five lines evaluated, the following occurred:

Table. 2.1. A list of the cultivars used during this research, with a brief summary of how the comparator swards for genotype and each treatment were developed.

Cultivar:	Bealey	LP258	LP256	R141	R164
Old Clonal Material:	24 fairy-ring grass structures for each line removed from paddock. ~20 tillers removed from each of the 24 sections. ~480 Tillers – 60 selected at random, and placed in each tray. Total of 6 replicates.				
New Material:	360 seeds selected at random – 60 in each tray. Total of 6 replicates.				

Cultivars or lines LP256, LP258, R141 and R164 are all diploid perennial ryegrass. LP256 was obtained from a parental crosses of Impact x European Varieties. It is described as having good seasonal yields, and high tiller density. LP258 was developed from a parental cross of Bronsyn x Spanish 403. LP258 has high yields, good persistence and is reportedly resistant to crown rust. R141 and R164 are recombinants. There is no information regarding their genetic lineage, performance or phenotypic traits. Lastly, Bealey, is a tetraploid perennial ryegrass with high yields, good palatability, and excellent seasonal growth.

Once the seedlings were established, the comparator swards were placed outside for 20 weeks until a trial site was developed.

2.2. Development of the Field Trial Site:

To develop a field trial site comprised of the old tiller derived and seed derived swards, a plot approximately 9mx6m was sown with turf grass. Once the turf grass was established, it was cut back and each of the swards were transplanted into the trial site as depicted in Figure 2.4. Each of the plots is comprised of two replicated trays. Thus, for each line or cultivar there was a total of three replicated plots for each treatment, specifically old or new. The field trial plan is shown in Table 2.2.

Figure 2.4. The field trial site.

Table 2.2. Field trial layout

LP256 O (25)	R164 O (26)	R141 N (27)	BEALEY O (28)	LP256 N (29)	R164 N (30)
LP258 N (19)	BEALEY O (20)	LP256 N (21)	LP258 O (22)	R164 N (23)	R141 N (24)
LP258 O (13)	R141 O (14)	LP258 N (15)	BEALEY O (16)	BEALEY N (17)	R164 O (18)
R141 O (7)	LP256 O (8)	BEALEY N (9)	R164 O (10)	R141 N (11)	LP258 O (12)
LP256 N (1)	R164 N (2)	R141 O (3)	LP258 N (4)	LP256 O (5)	BEALEY N (6)

The field site consisted of one single block divided into 30 plots comprised of 120 established seedlings (new) or tillers (old). Small numbers denote the plot number i.e. 1-30, and "N" or "O" indicates the treatment of either old or new. There are three biological replicates for each treatment per cultivar.

This experiment, including the development of the old and new comparator swards and a field site, adhered to a completely randomised design. A completely randomised design was appropriate for this experiment as the experimental units, specificially, the old and new comparator swards and the field trial site, were treated as unstructured, random, but homogeneous as they were developed in a standardized manner and were exposed to the same treatment for the duration of the experiment (Welham et al., 2014). Specifically, 60 of the old tillers and/or new seed were selected at random from ~480 tillers and/or ~360 seeds. These 60 tillers or seeds were then transferred at random into the swards, but as the swards were developed using a standardised tool, the density of each of the 6 replicated swards was equal,

confering homogeneity between the replicates. The allocation and/or treatment of the tillers or seeds for each of the respective lines being examined was homogenous. Thus, the development of ryegrass miniswards was unstructured but homogeneous and uniform.

Furthermore, the allocation and treatment of the miniswards in the field trial also adhered to a completely randomised design. Specifically, each of the swards were transferred into the field plot at random. Additionally, when sampling from the swards, each sward received equal treatment conferring homogeneity. Furthermore, it can be assumed that another source of potential homogenity in this experiment is the site or plot. To assess this, the yield of turf grass from around the plots was obtained, measured and compared via a one-way ANOVA. In the event that the yield of the surrounding turf grass was found to be significantly different (i.e. violates the assumption of homogeneity and uniformity), then post-hoc blocking factors were included in the analysis of the data. Conversely, if the yield of the turf was not signficantly different, then the data obtained from the experimental units could be analysed without blocking factors. The main advantage of a completely randomised design is that it is flexible, and if replication varies between treatments or if data is missing for some units, simple forms of statisical analysis can still be applied. To ensure that the field site was homogenous, as assumed during its development, turf grass surrounding each of the comparator plots was harvested and weighed utilizing the standardized method. However, the reel mower was lowered, as the turf grass was consistently maintained at lower levels throughout the experiment. Samples from the surrounding turf grass where obtained 32 weeks, 38 weeks and 44 weeks post development of the trial site. To obtain representative samples, turf grass from 5 different areas within the field trail site was harvested and compared. The different blocks are highlighted (A-E) in Table 2.3.

Table 2.3. Illustrates the areas of turf grass harvested to assess yield.

R164 O [26]		R141 N [27]		BEALEY O [28]		LP256 N [29]		R164 N [30]
	D						B	
BEALEY O [20]		LP256 N [21]		LP258 O [22]		R164 N [23]		R141 N [24]
				E				
R141 O [14]		LP258 N [15]		BEALEY O [16]		BEALEY N [17]		R164 O [18]
LP256 O [8]	C	BEALEY N [9]		R164 O [10]		R141 N [11]	A	LP258 O [12]
R164 N [2]		R141 O [3]		LP258 N [4]		LP256 O [5]		BEALEY N [6]

2.3 Standardized sampling and storage protocol:

To obtain samples for analysis of yield and persistence, and for the subsequent molecular component, a modified reel mower was used to harvest ryegrass 3cm above soil level. Between each cutting, the "catcher" was removed and cleaned. The grass cuttings were placed in bags labeled with the corresponding plot number, and were dried in the oven at 90^{o}c for 24-48 hours. When dried the grass was weighed. Prior to harvesting grass from each of the comparator plots, the surrounding turf grass was cut to a lower level than each of the plots, and residual turf grass from around the edges of the plots was cut using shears to minimize contamination of the samples. Samples for analysis of yield and persistence where obtained at 5-weeks, 10 weeks, 14 weeks, 24 weeks, 26 weeks, and 28 weeks post-transplanting, respectively.

Once yield data was obtained, it was subjected to statistical analysis. If the performance or dry matter production of old and new differed, it was necessary to then determine if differences in performance, specifically, yield and persistence, were linked to differences in viral load and/or multiple virus. However, the old and new ryegrass utilized during this project could potentially differ in three discernible ways:

- Genetic Drift - The old ryegrass material may be a genetically distinct subset of the original seed, a consequence of genetic drift or the production of genetic variation over time.
- Epigenetics – DNA methylation and/or histone modification overtime, which could alter or contribute to observable differences in the performance and/or productivity of old ryegrass when compared to new ryegrass. This alternative hypothesis may explain differences in performance or yield differences, if viral load does not correlate.
- Viral load, which according to previous research, could potentially increase overtime. It is therefore, likely that the viral load of old and new ryegrass will differ. Therefore, differences in the performance of old and new may potentially be attributable to viral load, and this is what this research aims to elucidate.

Technical Objective #2 – Detection and quantification of viruses:

In order to satisfy the second requirement of this research, specifically to ascertain if decreased ryegrass yield and persistence is potentially linked to viruses and/or viral load, it was necessary to determine if more than one virus was present in our ryegrass samples, and if the viral load or virus titre between old and new ryegrass actually differed. As previous literature indicates that ryegrass pastures could be infected with multiple viruses at any given point in time, and that viral load or the incidence of viruses within a pasture was likely to increase over time, it is plausible that more than one virus may be present within our ryegrass, and that the viral load of old ryegrass, which has been *in situ* for approximately 10 years, could be greater than the viral load of new or seed derived ryegrass (<1-year-old).

To facilitate the detection and quantification of viruses, a range of virus-detection methodologies were utilized during this project. Initially, ELISA (enzyme-linked immunosorbent assay) was conducted to determine if BYDV and RGMV was present in our material. However, ELISA was not used for quantification, nor could it be used to facilitate the detection of LPPV, as antisera for this virus is not available. In addition to ELISA, siRNA analysis was conducted. siRNA analysis exploits a natural cellular anti-viral defense system that is present in all eukaryotic organisms, known as RNA silencing (Boonham et al., 2014; Kreuze, 2014). This defense mechanism involves the targeted disintegration of double stranded viral RNA (dsRNA) (Boonham et al., 2014; Kreuze, 2014). dsRNA is detected and degraded by dsRNA-specific cellular Dicer-like endonucleases (Bi et al., 2012). Virus-derived siRNAs bind to ribonuclease H-like proteins within an RNA silencing complex (RISC), and are used to facilitate the detection and subsequent degradation of homologous RNA molecules. Degradation of viral RNA leads to the accumulation of small interfering RNA fragments 21-24 (nt) nucleotides in length with homology to the invading virus (Kreuze, 2014). siRNA analysis involves extraction, sequencing and de novo assembly of these small derived virus RNA fragments. These small RNA fragments are assembled into longer contiguous sequences (contigs), which are then used as queries in searches carried out in the NCBI database by BLAST, or specifically, nucleotide BLAST (BLASTN), to identify identical or highly homologous viral sequences (Kreuze et al., 2009; Wu et al., 2010; Bi et al., 2012). For this research, siRNA analysis was primarily used to confirm the presence of our target viruses, whilst facilitating the detection of previously unrecognized or novel viruses within our samples.

The predominant method utilized during this research was real time quantitative reverse transcription PCR (hereafter qRT-PCR). Whilst, qRT-PCR was initially developed for analysing gene expression, this method was readily adopted to faciliate the detection of viruses (Boonham et al., 2014). qRT-PCR is a highly sensitive technique that can be used to detect and/or quantify specific DNA/RNA transcripts with detection limits as sensitive as one transcript per 1000 cells (Marone et al., 2001; Lee et al., 2010). The most common method of qRT-PCR used for quantifying viral load and/or for the detection of viruses, involves the use of hydrolysis probes such as TaqMan® in combination with specific primers (Boonham et al., 2014). The TaqMan® method is based upon the 5' exonuclease activity of *Thermus aquaticus* (*Taq)* DNA polymerase and the fluorescent resonance energy transfer (FRET) activity of fluorophore reporter molecules and quenchers (Boonham et al., 2014). Essentially TaqMan® reagents incorporate the use of a fluorogenic probe to enable detection of a specific PCR product as it accumulates during each PCR cycle. Thus, the aim of this experiment was to develop a probe-primer assay. However, SYBR green, an intercalating dye, was used to initially validate the efficacy and efficiency of each of the PCR assays.

Whilst a range of virus detection methodologies are available, such as those aforementioned, qRT-PCR was the primary approach used during this research, as assays for viral detection and quantification can be developed, validated and implemented relatively quickly. Furthermore, once a generic base protocol has been developed and validated, it can be used to screen for multiple targets across a range of biological samples. However, it must be stated that all virus detection methodologies have limitations (Boonham et al., 2014). Specifically, ELISA, a serological based technique, which is the currently the industry standard for phytodiagnostics, was used to initially screen our ryegrass samples (Boonham et al., 2014). ELISA is designed to detect and quantify substances such as viral proteins (antigens) via the use of antibodies (Boonham et al., 2014). Whilst ELISA can facilitate detection of viral proteins in the range of 1-10ng virus/ml, the antisera or antibodies used in ELISA, often lack the resolution necessary to correctly distinguish between virus strains, which are closely related but have a distinct phenotype, as closely related strains tend to have conserved viral proteins, and thus relatively similar characteristics and properties (Boonham et al., 2014). In comparison to ELISA, molecular techniques can facilitate both specific and generic detection of viral isolates. However, molecular techniques, such as siRNA and qRT-PCR also have shortcomings. For example, for siRNA analysis to be most effective, particularly for the

identification of novel viruses, nucleotide sequences with a minimum level of homology must be present in the database to enable identification, but the process is not straightforward, as one must arbitrarily, define the upper and lower limits of similarity (E values >0.1), but lower E values or levels of similarity may present unspecific hits or uncharacterised retro-elements derived from the plant's own genome (Boonham et al., 2014; Kreuze, 2014). Lastly, qRT-PCR is most efficient when used in a generic manner. Specifically, the development of generic assays for viral detection and quantification enables one to detect a range of closely related viral strains or isolates within a single reaction event or biological sample. However, generic assays are developed upon the basis of a range of sequences with high degrees of similarity, and whilst that enables you to detect a range of closely related strains, you actually decrease the resolution of the assay overall. Specifically, you lose the ability to quantify the load or concentration of specific strains, and essentially, you can only quantify the combined load of all viral isolates (with similar sequences) within that biological sample. For example, our BYDV assay was designed to detect BYDV isolates PAV, MAV and PAS. However, as a generic assay was utilized to detect and quantify all of these particular BYDV isolates, we could not quantify the load of each particular isolate nor could we determine which isolate of BYDV may have had higher virus titre. To account for the limitations of each technique, it was necessary to use a range of detection methods. To conduct molecular analyses, the following methods were followed:

2.4. Sample acquisition & handling:

To obtain samples for viral detection and quantification, the standard sampling protocol outlined in section 2.3. was used, although with slight modifications. Specifically, a modified reel-mower was used to harvest the grass samples from each plot over 3 (15/4/15, 18/9/15 and 17/2/16) time points during 2015-2016. However, as opposed to being dried and weighed, fresh ryegrass tillers were stored at -80^{o}C, in accordance with the *Minimum Information* for publication of *Quantitative* Real-Time PCR *Experiments* guidelines (hereafter MIQE). To obtain a representative sample from each comparator plot per treatment and cultivar, samples obtained from each of the comparator plots were homogenized in liquid nitrogen, and ~5grams of crushed grass was transferred into 5 mL tubes, which were then stored at -80^{o}C.

2.5. RNA isolation and reverse transcription: From the plant material described, specifically, field grown ground grass samples, ~100mg (0.1g) of each sample was aliquoted into 2.0 mL snaplock tubes (Axygen®, NY, USA). RNA was extracted from each sample using Sigma Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) in accordance with the manufacturers protocol. However, once the required amount of 2-ME (2-Mercaptoethanol) lysis solution was transferred into each sample, a TissueLyser II® (Qiagen®, Redwood, CA, USA) was used in combination with stainless steel beads for 2 x 45 seconds, to obtain high-throughput disruption and homogenization of each of the respective samples. To ensure our experimental data was generated in accordance with MIQE guidelines, the integrity and quantity of extracted RNA was assessed (Taylor et al., 2010).

RNA was quantified using an Invitrogen Qubit® fluorometer with the Qubit® RNA buffer and dye (Invitrogen, Carlsbad, CA, USA). The Qubit® fluorometer was calibrated with the standards supplied by the manufacturer prior to use. The quality and/or integrity of the extracted RNA was analysed on a 1.5% denaturing formaldehyde agarose gel in 1x MOPS buffer, and then visualized on a BioRad Gel Doc (Bio-Rad, Hercules, CA, USA) by UV excitation of ethidium bromide (Sambrook and Russell, 2001). To ensure that intact RNA was obtained, images of the RNA gels were assessed to ensure that electrophoretic separation and UV excitation yielded distinct bands, indicative of small ribosomal RNA subunits 28S, 18S, 28S, and 1x 23S. At times, a microfluidic Bioanalyser (Agilent Technologies', Bioanalyzer, Bio-Rad Laboratories' Experion) system was used.

RNA purity was measured on a Nanodrop™ 1000 spectrophotometer in elution buffer (ThermoFisher Scientific, Waltham, MA, USA). The ratio of absorbance 260/280nm was used to assess the quality and purity of the extracted RNA. An OD ratio of >1.8-2.0 for each RNA sample was accepted as suitable for downstream applications. Ratios significantly lower than 2.0 indicate the presence of contaminants. By assessing the purity and quality of all biological samples, variability between samples can be reduced. Once isolated and checked, RNA was then stored at -80º. Following quantification and quality analysis, RNA was treated with Ambion® Turbo DNA-*free*™ DNase, (ThermoFisher, Scientific, Waltham, MA, USA) in accordance with the manufacturers protocol, to remove any potential genomic contamination from the RNA preparations. Prior to DNAse treatment, all RNA samples where diluted to 200 ng/50 µL in elution buffer. Following DNAse treatment, a Nanodrop™ 1000

spectrophotometer was used to quantify and reassess the purity of the RNA, to ensure that no genomic contamination was present.

To confirm that our RNA samples were free of genomic contamination, Endpoint PCR was performed on each RNA sample using plant specific primers, Eukaryotic elongation factor 1 alpha, hereafter eEF1A(s); 10 µM of eEF1A(s) forward primer (5'-CCGTTTTGTCGAGTTTGGT-3'), and 10 µM of eEF1A(s) reverse primer (5'-AGC AACTGTAACCGAACATAGC- 3') (Lee et al., 2010). For each 20 µL PCR reaction, the following components where included, 10x Kapa Tap Buffer 2.0 µL, 1.5 mM $MgCl_2$ (included in the Kapa Taq Buffer), 10 mM dNTP mix 0.4µl, 10 µM Forward Primer 0.4 µL, 10 µM Reverse Primer 0.4µL, 5 U/µL Kapa Taq DNA polymerase 0.1 µL, 4 µL of template, and 12.7 µL of PCR grade water. PCR reactions were performed in a GenePro Thermal Cycler (Bioer Technology, Binjiang District, China), according to the following parameters: initial denaturation 95^{o}C for 3 minutes x 1 cycle, followed by 35 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 60^{o}C for 30 seconds, extension at 72^{o}C for 1 minute, with a final extension cycle at 72^{o}C for 1 minute. PCR products were then visualised on a 3% agarose gel containing 2 µL or 0.02 mg mL^{-1} of ethidium bromide (Invitrogen, Carlsbad, CA, USA). Gels were run in ½ x Tris-borate-EDTA (TBE) buffer (89mM Tris, 44.5mM boric acid and 2mM EDTA pH and visualised by UV excitation of ethidium on a Bio-Rad GelDoc (Bio-Rad, Hercules, CA, USA). If genomic DNA was present within the DNAse treated RNA samples, one would expect to see an amplicon of 113bp, as depicted in Figure 2.6. Once assessed, RNA samples were quantified using an Invitrogen Qubit® fluorometer with the Qubit® RNA buffer and dye (Invitrogen, Carlsbad, CA, USA), as previously described. To ensure our results adhered to MIQE guidelines, it was necessary to quantify and reassess the integrity of RNA after DNAse treatment to minimize unwanted variation between samples (Bustin et al., 2009).

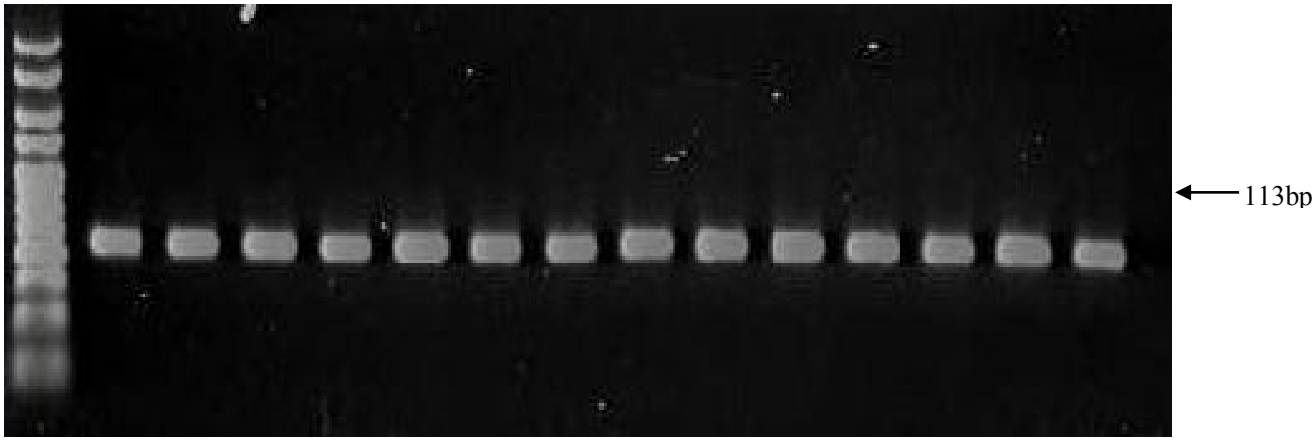


Figure 2.5. Gel image depicting cDNA amplified using eEF1A(s) primers

DNAse treated RNA was then used to synthesize complementary DNA (cDNA) using TaKaRa BluePrint™ RT-PCR kit (Takara Bio, Inc., Otsu, Shiga, Japan). For each sample, 350 ng of RNA was used in a 10 µL reaction with 0.5µL of Oligo dT Primer (50 µM), 0.5µL random hexamers (100 µM) and 2 µL of 5x PrimeScript Buffer, a total of 3.5 µL of the aforementioned components was aliquoted into each RNA sample (350 ng/µL) of *x* amount, and *x* amount of RNAse free dH_2O to ensure that each sample had a total reaction volume of 10 µL. cDNA was synthesized in accordance to the conditions specified by the manufacturer, specifically, 37 ^oC for 15 minutes (reverse transcription), 85 ^oC for 5 seconds (inactivation of reverse transcription with heat treatment), followed by a final step of 4 ^oC. Each cDNA sample synthesised according the manufactuers protocol was diluted 10-fold (1/10) with sterile PCR grade water and stored at -80 ^oC. To validate that the reverse transcription process was successful, all cDNA samples were checked via Endpoint PCR using plant specific primers, specifically, Eukaryotic elongation factor 1 alpha, eEF1A(s), and Endpoint PCR reaction components and conditions as previously described. cDNA was then stored at -20 ^oC until use in downstream qRT-PCR. The concentration of RNA reaction components, total RNA and thermo-cycling reactions conditions utilized during reverse transcription remained consistent throughout to minimize variability (Taylor et al., 2010). PCR products where visualized on a 3% agarose gel (containing 2 µL or 0.02 mg ml^{-1} ethidium bromide) via UV excitation of ethidium bromide on a Bio-Rad Gel Doc (Bio-Rad, Hercules, CA, USA), as previously described. cDNA that yielded a 113bp product, as depicted in Figure 2.5, were used for further experiments.

2.6. Primer Design: In order to design primers and/or probes for the detection of the target viruses, a preliminary alignment of all available nucleotide sequences on NCBI database was carried out using Geneious® 8.1.8 (Geneious® 8.1.8, Biomatters Ltd, USA). Once aligned, new primers were designed using the algorithm Primer3 Plus which is incorporated into Geneious®. Primers and probes where designed according to the specifications outlined in Table 2.4.

Table 2.4. Primer Specifications.

	Primers		Probe	
	Range	Ideal	Range	Ideal
Length	18-30	22	20-28*	24
Melting Temperature	60-64°C	62°C	66-70°C	68°C
GC content	35-65%	50%	35-65%	50%

Furthermore, target sequences were designed to amplify products 100-150bp long. Prior to being ordered from IDT (Integrated DNA Technologies Inc, San Diego, CA, USA) all prospective primers were subjected to thermodynamic analysis via IDTs OligoAnalyzer 3.1 (Integrated DNA Technologies Inc, https://sg.idtdna.com/calc/analyzer), to ensure that any potential secondary structures or homo-/heterodimers would not interfere with the PCR reactions.

2.6.1. Primers for BYDV: After a preliminary alignment of all available nucleotide sequences on the NCBI database, sequences that exhibited high variability were eliminated, and only sequences with highly conserved regions or regions with a high percentage of similarity (>80%) where used to develop primers, as listed in Table 2.5.

Table 2.5. A List of BYDV isolates, their origin, hosts and respective NCBI accession numbers.

Virus	Isolate	Origin of Isolate	Host	Accession no.
BYDV-MAV	BYDV-MAV-O1LU	New Zealand	*Avena Sativa*	GU002360
BYDV-MAV	BYDV-MAV-	New Zealand	*Triticum sp.*	GU002322
BYDV-PAV	BYDV-PAV-PC3	New Zealand	*Poa cita*	GU002329
BYDV-PAV	BYDV-PAV-WC2	New Zealand	*Triticum sp.*	GU002330

BYDV-PAV	BYDV-PAV-OA4	New Zealand	*Avena sativa*	GU002328
BYDV-PAV	BYDV-PAV-03LU	New Zealand	*Avena sativa*	GU002327
BYDV-PAV	BYDV-PAV-02LU	New Zealand	*Avena sativa*	GU002326
BYDV-PAS	BYDV-PAS-DC1	New Zealand	*Triticum sp.*	GU002323
BYDV-PAS	BYDV-PAS-DC2	New Zealand	*Triticum sp.*	GU002324

After which, Primer3 Plus was used to screen sequences as listed above, for prospective primers in accordance with the aforementioned specifications. Primer3 Plus identified the following primer pair:

Primer	**Forward 5' to 3' Sequence**	**Reverse 5' to 3' Sequence**	**Amplicon (bp)**	**Tm**
BYDV	CGCAATGCCCAGCGCTTTCAG	CGCAATGCCCAGCGCTTTCAG	124bp	60ºC

Thermodynamic analysis demonstrated that the ΔG (kcal.mole-1) value of all secondary structures was higher than -5kcal/mole, and that the secondary structure annealing temperature (T_m) was lower than the Tm of the primer pair, therefore, it was not likely that these secondary structures would interfere with downstream PCR reactions (Diefenbach & Dveksler, 2003). The generic primer set developed for detection and quantification of BYDV amplifies a conserved region within ORF3, which encodes the coat protein gene (Chomič et al., 2010). However, nestled within ORF3, is ORF4 which encodes the movement protein gene (Chomič et al., 2010; Ali et al., 2014). To facilitate detection and quantification of BYDV isolates PAV, PAS and MAV, and to validate the novel primer set, degenerate primers designed by Chomič et al., 2010 where initially used, as presented below.

Primer	**Forward 5' to 3' Sequence**	**Reverse 5' to 3' Sequence**	**Amplicon (bp)**	**Tm**
C1F1-C1R1	GGGGTMMTCAAATTCGGKCC	GAGTTCAATAAAKATWGCGCC	129	50ºC
C1F2-C1R2	TCGCAATGYCCAGCRCTTTCAG	AGCAAGTAAGTGGGCAGACAG	156	50ºC

Whilst the primers sets designed by Chomič et al., 2010, facilitated the initial detection of BYDV isolates PAV, PAS and MAV via Endpoint PCR, during subsequent qRT-PCR reactions, it was determined from melt curve analysis that these primers produced a secondary structure/PCR artefact or "primer dimer" that interfered with the PCR reaction, and impeded our capacity to accurately detect and/or quantify BYDV. After which, only the novel primers

sets developed for this research where used during subsequent qRT-PCR reactions.

2.6.2. Primers for RGMV: An alignment of all available nucleotide sequences on the NCBI databased was performed using Geneious®8.1.8. Again, only sequences with high levels of similarity where used to develop primers, as listed in Table 2.6.

Table 2.6. A list of RGMV isolates, their origin, host and respective NCBI accession numbers.

Virus	Isolate	Origin of Isolate	Host	Accession no.
RGMV	A-V	Australia	*Lolium perenne*	AF035818
RGMV	Otago	New Zealand	*Lolium perenne*	AF901243
RGMV	ACT	Australia	*Lolium perenne*	AF035639
RGMV	RGMV-B	Wales (U.K.)	*Lolium perenne*	AF035640
RGMV	Kaikohe	New Zealand	*Lolium perenne*	AF091244
RGMV	Ger	Germany	*Lolium perenne*	AJ889241
RGMV	S. African	South Africa	*Lolium perenne*	RMU27383

Whilst adhering to the same primer specifications, Primer3 Plus identified the following primer set:

Primer	Forward 5' to 3' Sequence	Reverse 5' to 3' Sequence	Amplicon (bp)	Tm
RGMV	GCTTCATGGTTTGGTGCATGG	GTGCCATTATTGACCGCAACG	144bp	58^{o}C

This primer set was subsequently analyzed as previously described. The results of the thermodynamic analysis indicated that secondary structures had ΔG (kcal mol^{-1}) values higher than -5, and annealing temperatures (T_m) lower than that of the primers themselves. Therefore, these structure should not interfere with PCR reactions. These primers amplify a conserved region within the coat protein sequence, and where used to detect and quantify RGMV.However, to facilitate the initial detection of RGMV isolates, whilst validating the novel primer sets, primers designed by Webster et al., 1996, where used, as presented below.

Primer	Forward 5' to 3' Sequence	Reverse 5' to 3' Sequence	Amplicon (bp)	Tm
RGMV - 1	GAACATGACTTCCACGACATCACCGACA	AGCAAGTAAGTGGGCAGACAG	950bp	55^{o}C

2.6.3. Primers for LPPV: Currently, there are no available nucleotide sequences in the NCBI database for this particular virus. As such, sequences of viruses within the family *Partitiviridae* and genus *Deltapartitivirus* were screened and used to develop degenerate primers that would amplify conserved sequences, such as nucleotide sequences that encoded RdRp and the coat protein. However, in the absence of positives, the efficacy of these primers to detect LPPV could not be confirmed.

2.6.4. Validation of Primers: To ensure that primers from the literature and/or the newly developed primers amplified the correct sequences, Endpoint PCR was performed on the cDNA synthesized from the ryegrass samples using each of the aforementioned primer sets for BYDV (F – CGCAATGCCCAGCGCTTTCAG; R – CGCAATGCCCAGCGCTTTCAG), and RGMV (F - GCTTCATGGTTTGGTGCATGG; R – GTGCCATTATTGACCGCAACG). PCR products amplified using novel BYDV and RGMV primers were visualised on a 3% agarose gel (containing 2 μL or 0.02 mg mL^{-1} ethidium bromide) via UV excitation of ethidium bromide on a Bio-Rad Gel Doc (Bio-Rad, Hercules, CA, USA), as depicted in Figure 2.6 and 2.7.

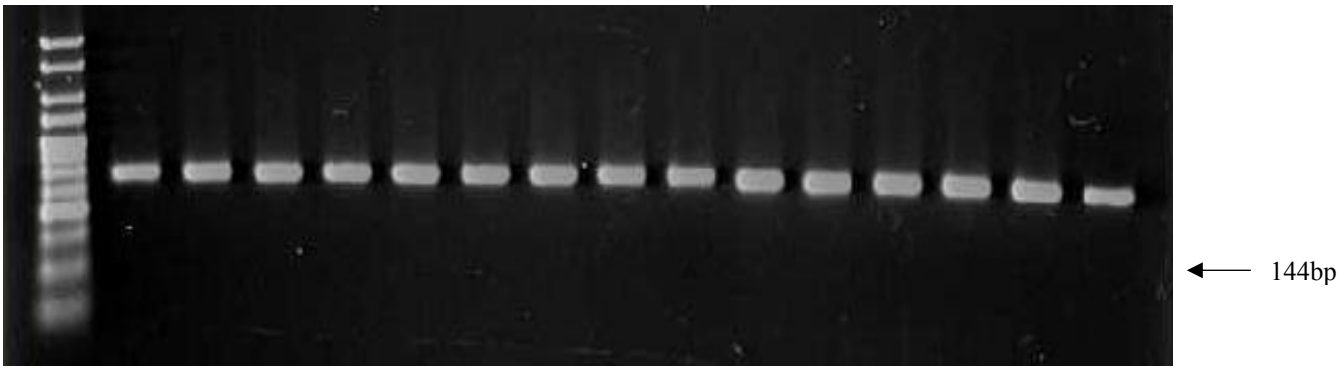


Figure 2.6. Gel depicting PCR products amplified via novel RGMV primers

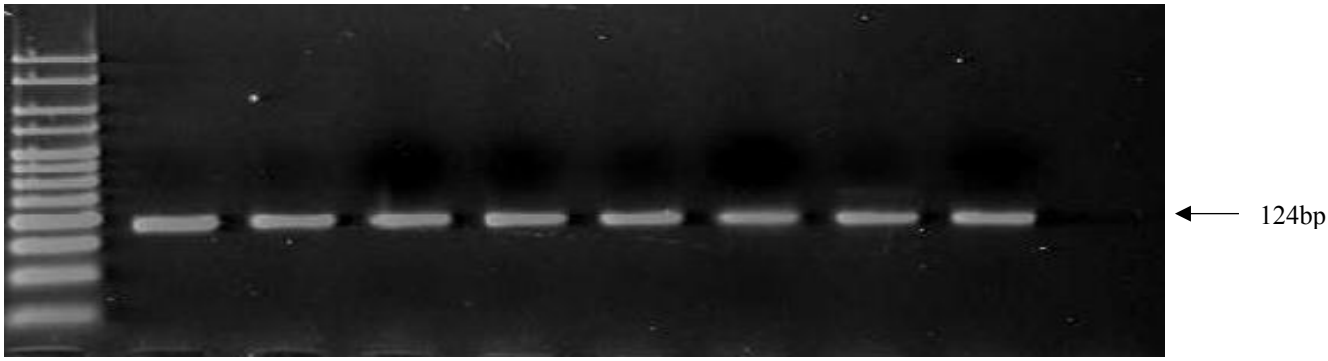


Figure. 2.7. Gel depicting PCR products amplified via novel BYDV primers.

PCR products were then extracted using a NucleoSpin® Gel Extraction and PCR clean up kit in accordance with the manufacturers protocol (Machery-Nagel, Düren, Germany). The extracted products were quantified via a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA) as previously described in section 2.5. The extracted PCR products were analyzed at Lincoln University's Sequencing facility. Sequences were then blasted via Geneious® 8.1.8 to ensure that the novel and/or existing primers sets amplified the correct sequences. Once primers had been validated, and their capacity to detect the correct sequences and/or target genes had been confirmed, PCR products obtained from subsequent Endpoint PCR reactions were used as positive controls during qRT-PCR and to generate standard curves for each assay.

2.7. qRT-PCR: During the course of this research, quantitative qRT-PCR was used to quantify viral nucleic acids. Quantitative reverse transcription PCR involves the use of standard curves to quantify target transcripts within a given sample through interpolation from the standard curve. A standard curve was generated by creating a 10-fold dilution series across multiple $\log_{10}$ concentrations.

2.7.1. Establishment of a Standard Curve: Prior to qRT-PCR analysis, a PCR product of known concentration (1 ng/µL) was used to create a dilution series across multiple $\log_{10}$ concentrations, and to generate an 8-point standard curve for each assay. The dilution series covered 7 orders of magnitude (1×10^{-1} - 1×10^{-8}). To perform the 10-fold dilution series, Eppendorf epMotion 5070 liquid handling robot (Eppendorf Co., Hamburg, DEU, Germany) was used.

Each dilution of known concentration serves as a standard, and each of these standards was subsequently tested via qRT-PCR. To achieve this, Eppendorf epMotion 5070 liquid handling robot (Eppendorf Co., Hamburg, DEU, Germany) was used to pipette 4 µL from each standard, and 6 µL of master mix containing 0.4 µL (0.2 µL forward and 0.2 µL reverse) of the qRT-PCR primers, 0.6 µL PCR grade water, and 5 µL 2x TakaRa SYBR® Premix ExTaq ™ II PCR reagents, a non-specific intercalating dye (TaKaRa Bio, Inc., Otsu, Shiga, Japan) into each of the 10 µL wells on a 48-well plate. qRT-PCR analysis of the dilution series was performed in triplicate for all primer pairs using Illumina Eco™ Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). Standard thermocycling parameters where used throughout each assay, specifically, 95°C for 1 minute x 1 cycle, followed by 35 cycles of

denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 15 seconds, followed by melt curve analysis with denaturation at 95°C for 15 seconds x 1 cycle, annealing at 55 °C for 15 seconds x 1 cycle and denaturation 95°C for 15 secs All assays included a final incubation stage of 30°C for 30 seconds.

The Illumina EcoStudy 4.0 software (Illumina, Inc., San Diego, CA, USA) generates a standard curve, in which the mean Cq (*quantification cycle*) value of each standard is plotted against the log (quantity) or concentration of the standards. Cq is the PCR cycle at which flourescence or amplification crosses the detection threshold level. The time at which amplication is first detected above the threshold, is related to the inverse log of the quantity of the target being amplified (Boonham et al., 2014). Thus, the lower the Cq value for a sample the greater the starting amount of DNA transcript in the sample.

On the basis of the standard curve, Illumina EcoStudy 4.0 software calculates the performance and efficiency of each PCR reaction, and determines the analytical sensitivity or the limits of detection/quantification of the assay. Ideally, amplification of a series dilution will produce a linear standard curve, with a reaction efficiency in the range of 90%-110% and a performance r^2 value >0.99. Standard curves for each assay developed during this research are included in Appendix 3.

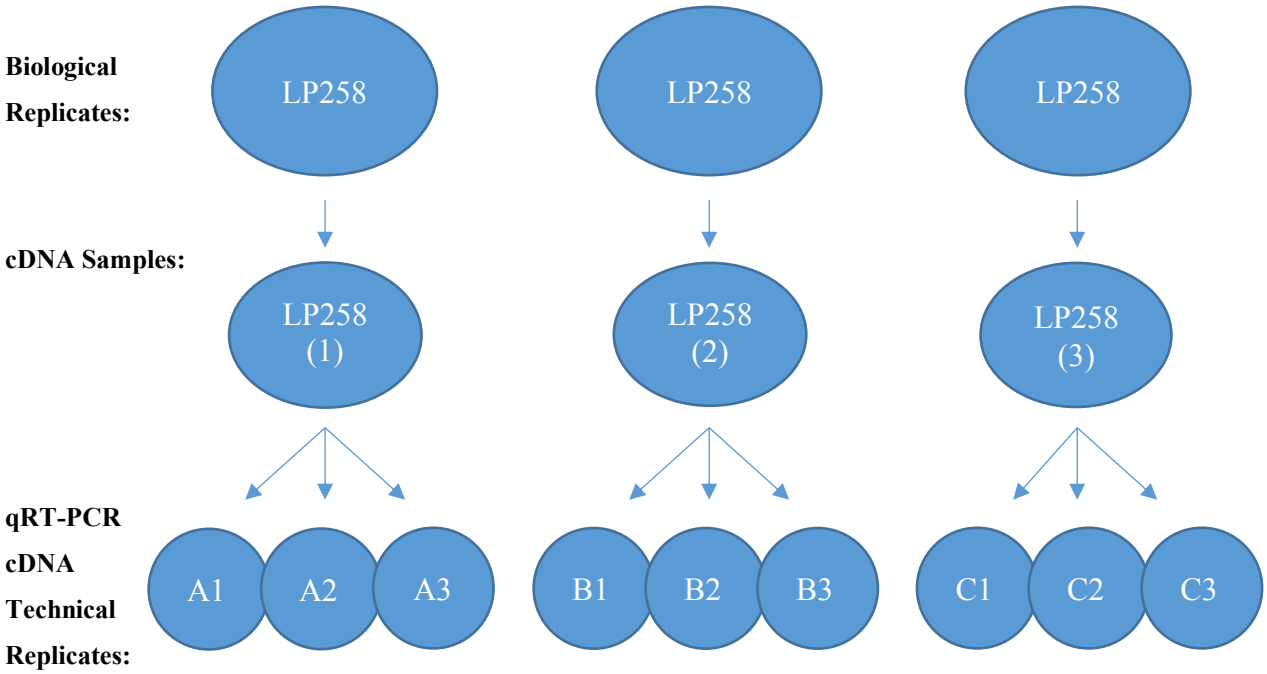
Furthermore, the range of dilution over which the standard curve is developed defines the limits of detection and/or quantification, or the analytical sensitivity of the assay. For example, for BYDV, an 8-point standard curve was developed. According to the standard curve, the lowest concentration of viral transcript or most dilute sample that was detectable with reasonable certainty was 0.00001ng (LOD), and amplification measured within the in the range of 7-24 Cq (LOQ) could be quantified with reasonable accuracy. However, amplification measured outside the range of 7-24 Cq could not be quantified accurately with high levels of confidence, and thus, samples outside of this range become non-quantifiable. This does not suggest that BYDV is not present within the samples, only that at concentrations <0.00001 ng or beyond 10^{-5}, BYDV cannot be reliably or accurately quantified by our assay. Once standard curves had been obtained for each assay, qRT-PCR analysis was performed across the ryegrass samples.

2.7.2. qRT-PCR assays: qRT-PCR assays were performed on cDNA synthesized from ryegrass tillers as described above, using an Illumina Eco™ Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). For each assay, 6 µL of master mix containing 0.4 µL (0.2 µL forward and 0.2 µL reverse) of the qRT-PCR primers, 0.6 µL PCR grade water, and 5 µL 2x TakaRa SYBR® Premix ExTaq ™ II PCR reagents, a non-specific intercalating dye (TaKaRa Bio, Inc., Otsu, Shiga, Japan) and 4 µL of cDNA template was aliquoted into each of the 48-wells on the qPCR plate using an Eppendorf epMotion 5070 liquid handling robot (Eppendorf Co., Hamburg, DEU, Germany) to reduce pipetting error. Thermocycling conditions adhered to those previously described in section 2.7.1. As an intercalating dye, specifically, SYBR® was used during each qRT-PCR reaction, it was necessary to conduct a melt curve analysis at the end of each cycle to confirm the specificity of the primers and to check for non-specific amplification.

2.7.3. Replicates & Controls: During this experiment, 5 different lines of ryegrass where examined. For each line, there are two treatments, specifically old and new, and for each treatment there are three biological replicates. To mitigate the effect of biological and technical variability, and to permit statistical analysis, at least three biological and three technical replicates per biological replicate should be included in each experiment.

Essentially, for each biological replicate (3) there will be three technical replicates (3), therefore, 9 individual qPCR reactions. During each assay, triplicates of positive standards were included in the first plate. Each plate also contained a triplicate of plate calibrators (PC) or inter-run calibrators, which are identical samples that are placed in the exact same wells on each plate in triplicate during each run. Plate calibrators are required, as they normalize inter-plate variation, whilst acting as positive controls to ensure that the reaction mix is working. Lastly, each plate contained no-template controls (NTCs) in triplicate. NTCs contain the same master mix as the other samples, but PCR grade water in place of DNA. No template controls are used to test for genomic contaminaton and PCR artefacts, such as primer-dimers. NTCs should not registers a Cq value. The following diagram illustrates the biological replicates and technical replicates used during this research, and the design or layout of the first 48-well plate used in each reaction (Figure 2.8). Subsequent plates contained only triplicates of plate calibrators, NTCs and samples. Specifically, for sample LP258, there are 3 different biological replicates for each treatment (old and/or new). From the tillers obtained from each

biological replicate, RNA was extracted and cDNA was synthesised. From each cDNA sample, 3 x 4 µL of cDNA was aliquoted into each three wells on each plate, providing three technical replicates. The NTCs contained 4 µL of PCR grade sterile water.



qRT-PCR First qRT-PCR Plate Layout

	1	2	3	4	5	6	7	8
A	10^{-2}	10^{-2}	10^{-4}	10^{-4}	10^{-6}	10^{-6}	10^{-8}	10^{-8}
B	10^{-2}	10^{-2}	10^{-4}	10^{-4}	10^{-6}	10^{-6}	10^{-8}	10^{-8}
C	10^{-2}	10^{-2}	10^{-4}	10^{-4}	10^{-6}	10^{-6}	10^{-8}	10^{-8}
D	LP258 A1	LP258 A2	LP258 A3	LP256 A	LP256 A	LP256 A	NTC	NTC
E	LP258 B1	LP258 B2	LP258 B3	LP256 B	LP256 B	LP256 B	NTC	PC
F	LP258 C1	LP258C C2	LP256 C3	LP256 C	LP256 C	LP256 C	PC	PC

Figure 2.8. The experimental replicates, and 48-well plate layout.

Once qPCR data was obtained for each of the respective assays, the Cq values obtained were converted to copy number before the data could be normalised.

2.7.4. Converting Cq Values to Copy Number: Raw data obtained from qRT-PCR assays representing quantification cycle or Cq values was exported from Illumina EcoStudy 4.0 Software (Illumina, Inc., San Diego, CA, USA) to Excel. In Excel, another standard curve was constructed by plotting the log quantity of each standard against the mean Cq values obtained from the technical replicates. To convert the mean Cq value of each standard to an exact concentration in nanograms and then to copy number the following approach was used. For example, for BYDV the graph efficiency was: $y = 1.487 + 7.4871$, as depicted below in Figure 2.9, and the mean Cq value was *10* at 0.1 ng/uL as listed below in Table 2.7.

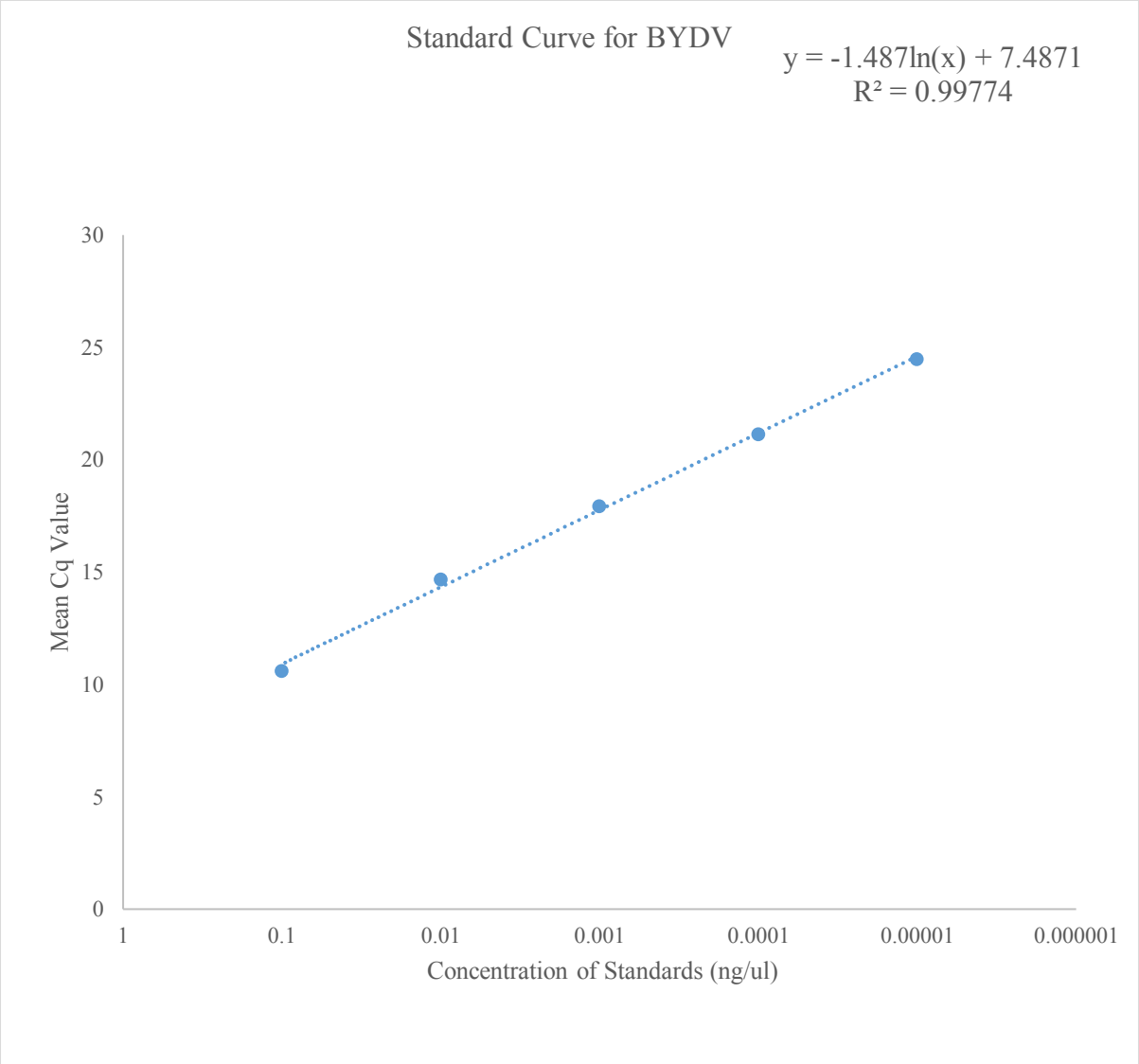


Figure 2.9. Illustrates the standard curve obtained for BYDV.

To obtain a value in nanograms from the Cq mean, the graph efficiency equation was rearranged to obtain: $x = exp((7.4871 - y)/1.487)$. Where "*y*" = the Cq mean, in this case, 10.

Table 2.7. Lists the Cq means and copy numbers of BYDV Standards.

Assay	Cq mean	ng/ul	Graph effeciency	(ng)	(ul/ng)	(ng) x 10	Copy Number
BYDV	10.58716179	0.1		0.124334979	0.031083745	0.310837446	2287260736
BYDV	14.67538144	0.01	y = 1.487+ 7.4871	0.007953979	0.001988495	0.019884947	146321037.6
BYDV	17.91863461	0.001	ln(x)=(7.4871-y)/1.487	0.000898167	0.000224542	0.002245418	16522642.31
BYDV	21.13704426	0.0001	**x=exp((7.4871-y)/1.487)**	0.00010313	2.57825E-05	0.000257825	1897177.86
BYDV	24.47582775	0.00001		1.09209E-05	2.73022E-06	2.73022E-05	200900.0792

By using the equation, $x = exp\left(\frac{7.4871-10}{1.487}\right)$, the value in nanograms can be determined, specifically, 0.12ng. Nanograms or "0.12ng" was then divided by 4 (the amount of cDNA template used during the qPCR reaction), to obtain 0.031(ng/µl). *X*(ng/µl) was then multiplied by 10 to generate the amount in ng/10µl, as 10µl was the total reaction volume used during amplification. Multiplying 0.031(ng/µl) x 10 = 0.31ng/10µl. The amount of ng/10µl or 0.31ng/10µl is the value which is then converted into copy number using the following formula:

$$Number\ of\ Copies\ (Molecules) = \frac{X(ng) * 6.0221x10^{23} molecules}{\left(N * \frac{660g}{mole}\right) * 1x10^{9} ng/g}$$

*Where, *X* = amount of template in ng/10 µl, *N* = length of the dsDNA amplicon, *660g/mole* = the average mass of 1bp dsDNA and $6.0221x10^{23}$ is Avogadro's constant. This formula was adapted from Integrated DNA Technologies Inc. (https://sg.idtdna.com/pages/decoded/decoded-articles/pipet-tips/decoded/2013/10/21/calculations-converting-from-nanograms-to-copy-number).

The aforementioned equations and approaches were used to converted all mean Cq values into non-normalised copy number, as listed in Appendix 4 and Appendix 5. Once a copy number for each sample was obtained, it was normalized.

2.7.5. Normalisation of qPCR data: In order to generate reliable qRT-PCR data, it is essential to develop and validate an appropriate normalization strategy to mitigate the impact of variation between biological samples (Bustin et al., 2009; Lee et al., 2010; Tashiro et al., 2015). Variation between samples can be introduced at any stage throughout the experimental process or it can arise due to inherent differences in biology (Taylor et al., 2010). The most

common approach used to account for inter-sample variation or to normalize qRT-PCR data, such as copy numbers, involves the use of one or more endogenous reference genes that exhibit stable expression, irrespective of biological or physiological state (Bustin et al., 2009; Lee et al., 2010; Tashiro et al., 2015). Prior to analyzing the expression or transcript abundance of your target genes, it is necessary to validate reference genes to ensure that they have constant expression profiles across all of your samples and treatments.

To obtain suitable references genes for perennial ryegrass, literature was reviewed and on the basis of this, two references genes were selected and utilized. Specifically, eukaryotic elongation factor 1 alpha, eEF1A(s) and TAT-binding protein homolog 1, TBP-1, as designed and validated by Lee et al., 2010. To validate the expression stability of these reference genes, Cq values obtained from qRT-PCR analysis where converted into copy number, as previously described in section 2.8.4. Once converted, the non-normalized copy number data for each reference gene was exported from Excel into geNorm 3.4 Excel applet. The geNorm software determines the pairwise stability value or "*M*" value of each reference gene, by comparing the stability of that gene to stability of the other genes being examined (Tashiro et al., 2015). Genes with the lowest *M* value, $M < 0.5$ have the most stable expression. geNorm calculates a gene expression normalization factor for each sample based upon the geometric mean of the two reference genes. Normlisation data for the reference genes is documented in Appendix 5. The non-normalised copy number values for all samples obtained during this research were subsequently divided by the respective normalization factor generated by geNorm, as documented in Appendix 6. Once normalized copy number values where obtained the data was then exported to GenStat software version 18 for statistical analysis.

2.7.6. Statistical Analysis of qRT-PCR data: Normalized copy number values obtained from each virus assay, specifically, RGMV and BYDV, were subjected to a base-10 log transformation prior to statistical analysis, in order the satisfy the assumptions of a parametric statistical test, such as ANOVA. A repeated measurement ANOVA was conducted by David Baird via GenStat version 18 (VSN International, Ltd.), to determine if there were significant differences in viral load over time, between each treatment and between each of the cultivars examined. REML ANOVA analysis was adjusted for correlations. For BYDV, as many samples where outside the limits of detection and/or quantification, non-quantifiable data was adjusted by adding ½ the minimum value of the lowest copy number detected (Appendix 5).

P-values <0.05 were considered to be statistically significant. Graphs reflecting the results of the REML ANOVA were generated using GenStat 18 software (VSN International, Ltd.).

2.8. ELISA: ELISA assays were carried out by John Fletcher at Plant & Food Research. using an Agdia (Agida Inc., Indiana, USA) compound ELISA kit in accordance with the manufacturer's instructions. A positive reaction "cut-off" was calculated by adding the mean optical density of the 6 controls to 3 times their standard deviation.

2.9. siRNA: To obtain biological material for siRNA analysis, a representative sample of tillers was obtained from 10 plots for each of the 5 cultivars. These tillers where kept on ice and transported to Plant and Food Research, Lincoln. siRNA extraction and analysis was carried out by Sandi Keenan at Plant & Food Research. However, only two cultivars per treatment, specifically, Bealey and LP258 old and new, where analyzed. The bioinformatics software used to analyze siRNA data was Yabi (Murdoch University, Centre for Comparative Genomics, 2006-2016: http://ccg.murdoch.edu.au/yabi.). With *E*-values set at 0.05. Once complete, contigs where blasted via Geneious® 8.1.8. Only "hit" sequences with highly conserved regions or regions with a high percentage of similarity (>85%) were accepted.

The methods outlined above generated the following results.

Chapter Three

Results – Technical Objective #1:

3.1. Field Site & Turf Grass Yield: From analysis of the field trial site to test for homogeneity, the following yield data (grams of dry matter) was obtained from each of the turf grass plots.

Table 3.1. Turf grass yield data.

	DM Yield (grams)		
Plots	**18/02/16**	**24/03/16**	**5/05/16**
A	45.12	25.1	40.3
B	44.21	23.91	41.8
C	47.09	27.9	40.4
D	49.1	28.11	42.1
E	46.26	24.91	39.7

To test for variation within the trial site that could potentially contribute to variation between the ryegrass plots themselves, a general ANOVA was conducted using the data listed in Table 3.1. The general ANOVA of the surrounding turf grass plot indicated that the yield surrounding the turf grass or new and old comparator plots was not significantly different. Consequently, blocking factors were not required for further ANOVA analyses. Turf yield data is listed in Appendix 1, whilst the ANOVA output is included in Appendix 2.

3.2. Ryegrass Yield: From the agronomic data obtained from the new and old comparator ryegrass plots during 2015-2016, a general ANOVA was conducted to determine if there was a significant difference between the yield of old and new ryegrass. Full data output is listed in Appendix 1. To illustrate the results of the general ANOVA, the following charts were developed.

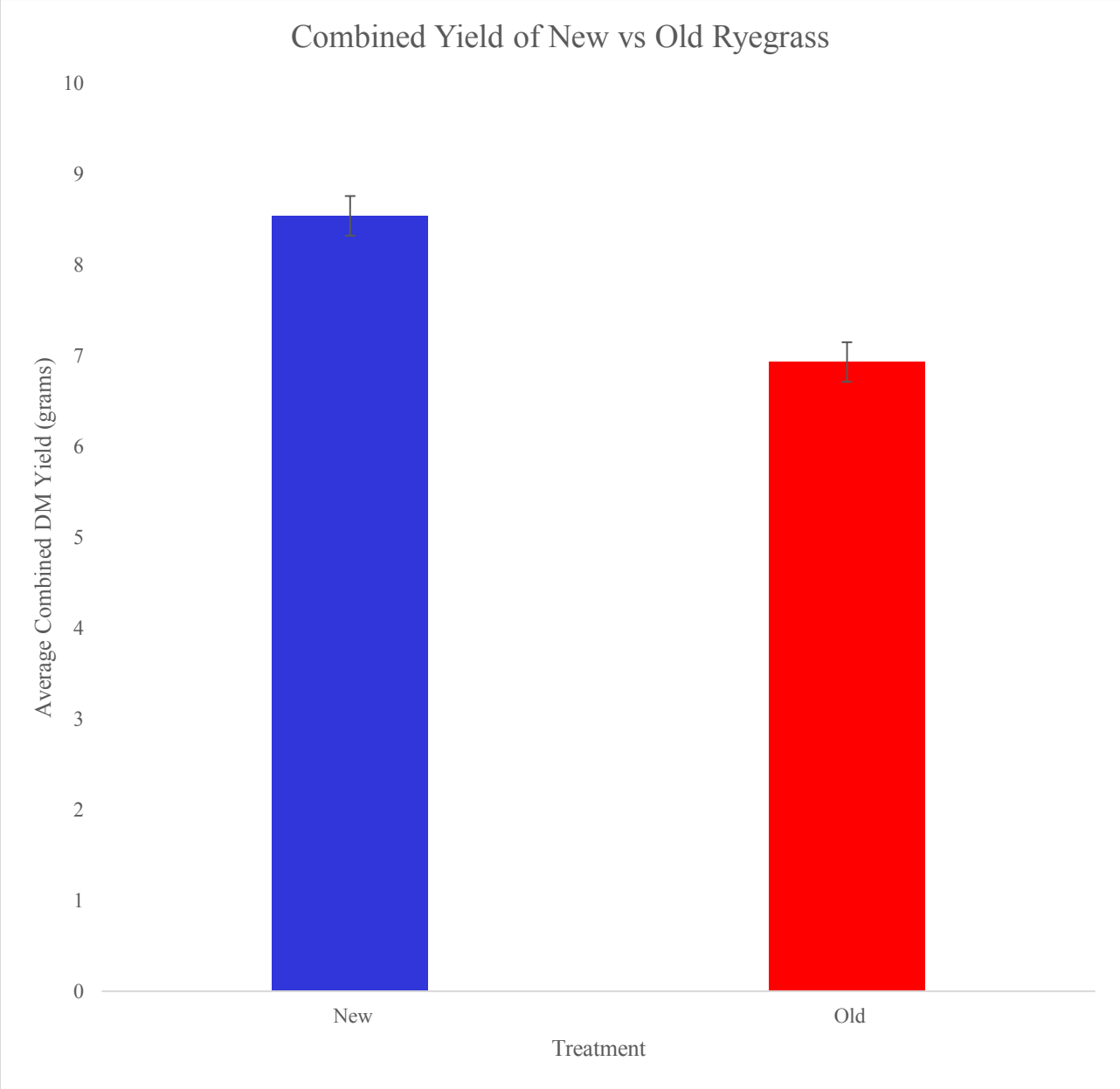


Figure 3.1. The combined mean yield for each treatment (P<0.01; df 1; SED = 0.218; LSD = 0.431).

From the results of the one-way ANOVA, it can be observed that there is a significant difference in yield between old and new ryegrass, as depicted in Figure 3.1. For the yield between old and new ryegrass to be significantly different, the combined mean yield values must exceed the LSD. In this case, as the mean yield values between old and new ryegrass are greater than the LSD of 0.431, the results are confirmed as significant. Specifically, the yield of new ryegrass is significantly greater than the yield of old ryegrass.

Additionally, the results indicate that there is a significant difference in mean yield production between old and new ryegrass for each genotype examined (Figure 3.2). However, for the yield of old and new ryegrass for each cultivar or line to be significantly different, the mean values must exceed the LSD of 0.946. For all cultivars examined, except LP258, the mean yield value between old and new ryegrass exceeds the LSD, thus the yield of new ryegrass for each of those cultivars is significantly higher than the yield of the older counterpart (Figure 3.2). However, the mean yield value between old and new ryegrass for cultivar LP258 did not exceed the LSD. In other words, the yield between LP258 old and LP258 new, whilst different, was not significantly different.

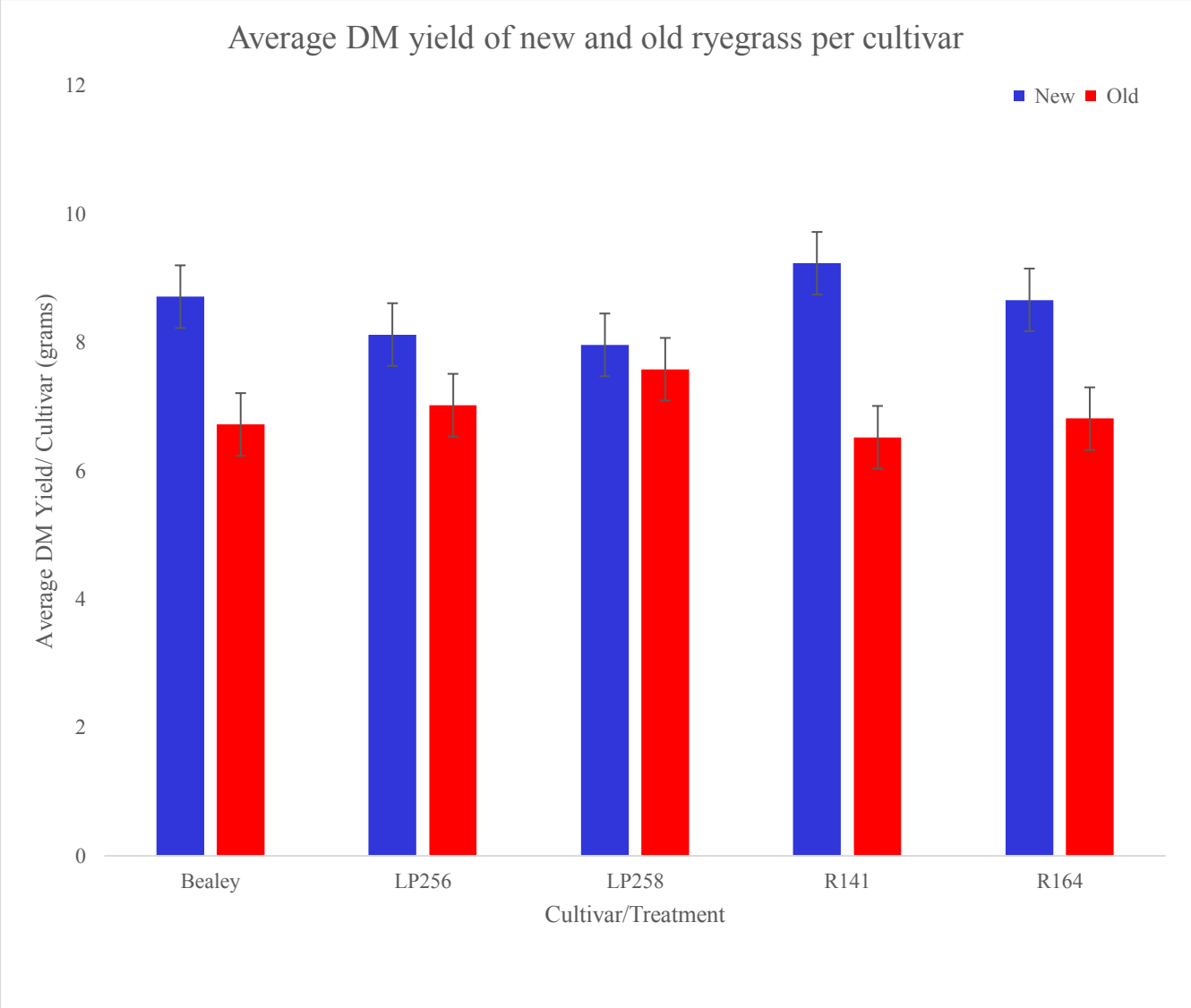


Figure 3.2. The combined mean yield of each cultivar per treatment (P<0.05, df 4; SED = 0.448; LSD = 0.946).

From the agronomic data it was determined that yield between old and new ryegrass differed depending upon genotype or ryegrass line. The differences between old and new ryegrass per line are summarized in Table 3.2.

Table 3.2. A list of the combined mean yield values for old and new ryegrass for each cultivar.

Cultivar/Line	Yield of New (grams)	Yield of Old (grams)	Difference in yield (%)
Bealey	**8.71**	**6.72**	**25.77**
LP256	**8.12**	**7.02**	**14.53**
LP258	**7.96**	**7.58**	**4.89**
R141	**9.23**	**6.52**	**34.41**
R164	**8.66**	**6.81**	**23.91**

3.3. Ryegrass Persistence: To analyze persistence, all agronomic data obtained for each treatment, old or new, per line was combined and compared over time to generate the following line plot (Figure 3.3). The line plot demonstrates that the yield of old and new ryegrass for each cultivar varied over time, perhaps a consequence of environmental interactions and/or seasonal variation. Furthermore, Figure 3.3 shows that overall there is a downward trend in dry matter production or yield over time for each cultivar examined, except LP258. Figure 3.3 demonstrates that LP258 appears has consistent or comparable yield performance over time irrespective of treatment or "age". Lastly, from Figure 3.3 it can be seen that overall the ten-year-old ryegrass material examined had a lower mean yield (grams of dry matter) throughout this project, in comparison to the new ryegrass material examined.

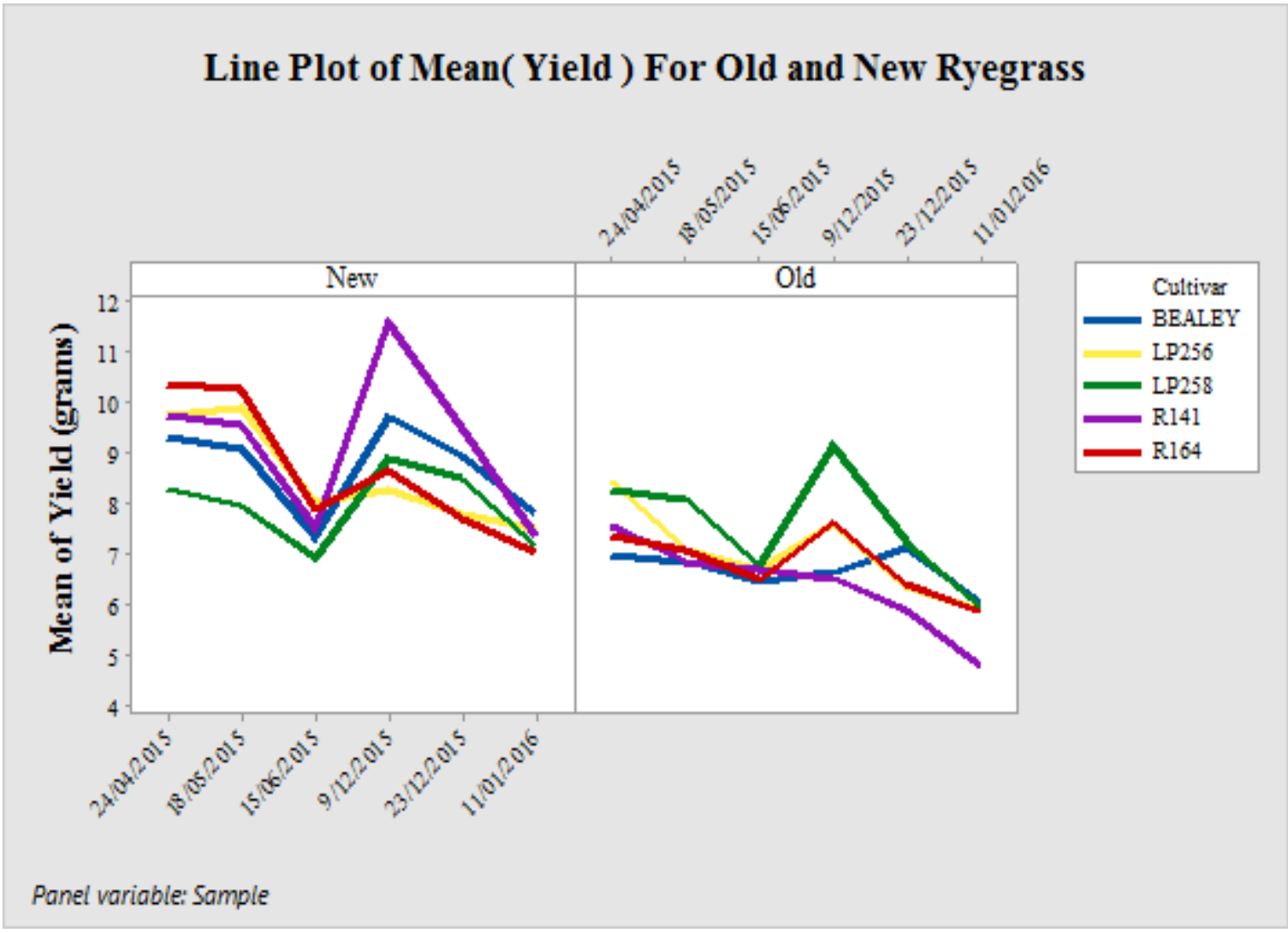


Figure 3.3. Yield over time for each cultivar and treatment

Overall, the agronomic data obtained and analyzed indicates that 1) the yield of young or new ryegrass greatly exceeds that of old ryegrass, 2) that ryegrass yield and/or yield stability is influenced by environmental and/or seasonal variation, and 3) that different cultivars exhibit different levels of persistence or yield stability over time. Whilst the data and statistical analysis indicates that there is a difference between old and new ryegrass with respect to yield or dry matter production (grams), and potentially yield stability or performance overtime, Figure 3.4 clearly illustrates the difference between old and new with respect to quality and/or performance.

Figure 3.4. Old and new comparator swards for each line.

Results – Technical Objective #2:

3.4. ELISA: The results of the preliminary ELISA assay of old ryegrass tillers indicates that BYDV isolate MAV, and RGMV were present in all cultivars, as summarized in Table 3.3. These results indicate that at the beginning of the project, all old ryegrass tillers were co-infected with, BYDV-MAV and RGMV. Results are presented as ELISA index i.e. mean OD divided by mean OD control.

Table 3.3. A list of viruses detected in old ryegrass tillers by ELISA.

	BYDV		CYDV	RGMV
Cultivar/Treatment	**PAV**	**MAV**	**RPV**	
Bealey Old	0	1	0	13.2
R164 Old	0	1.06	0	3.7
LP256 Old	0	1	0	5.8
LP258 Old	0	1.06	0	4.9
R141	0	2.3	0	24.6

A subsequent ELISA assay was performed using samples obtained from both new and old ryegrass tillers for each cultivar. However, during the second ELISA assay, only BYDV was analyzed. From these results, it can be observed that BYDV isolate PAV was detected in two of the old samples, specifically LP256 and R141, as presented in Table 3.4. BYDV-PAV was detected in all new samples except for LP256. No other BYDV isolates were detected in the ryegrass tillers. However, BYDV- MAV and CYDV-RPV were detected in R164 new.

Table 3.4. A list of viruses detected in new and old ryegrass tillers by ELISA.

	BYDV		CYDV
Cultivar/Treatment	**PAV**	**MAV**	**RPV**
Bealey Old	0	0	0
R164 Old	0	0	0
LP256 Old	1.29	0	0
LP258 Old	0	0	0
R141 Old	1.17	0	0
Bealey New	1.29	0	0
R164 New	1.27	1.1	1.27
LP256 New	0	0	0
LP258 New	1	0	0
R141 New	1.52	0	0

3.5. siRNA: The results of siRNA BLASTN analysis are summarized in Table 3.5. and Table 3.6.

Table. 3.5. BLASTN summary of contigs obtained from Bealey old and new.

Sample	NCBI Accession Number	Virus/Isolate	Average %	Contig Hits #
Bealey Old 21-25nt				
	AF035637	Ryegrass mosaic virus strain RGMV-AV	99.72	3
	AF191073	Stealth virus 1 clone 3B43,	92	1
	AF035818	Ryegrass mosaic virus strain RGMV-AV	95.52	10
	Y09854	Ryegrass mosaic virus	90.47	8
	KR061300	Ryegrass mosaic virus isolate Denmark	91.65	10
Bealey Old 21-22nt				
	AF035638	Ryegrass mosaic virus strain RGMV-3	98.72	1
	Y09854	Ryegrass mosaic virus, complete genome	90.83	8
	KR061300	Ryegrass mosaic virus isolate Denmark	92.47	10
	AF035639	Ryegrass mosaic virus strain RGMV-5	96.41	2
	AF035818	Ryegrass mosaic virus strain RGMV-AV	96.13	10
Bealey Old 24nt				
	AF035639	Ryegrass mosaic virus strain RGMV-5	97.27	1
	Y09854	Ryegrass mosaic virus	90.86	9
	KR061300	Ryegrass mosaic virus isolate Denmark	91.91	11
	AF035818	Ryegrass mosaic virus strain RGMV-AV	95.58	11
	AF035637	Ryegrass mosaic virus strain RGMV-AV	99.46	3
	AF065755	Stealth virus 1 clone 3B43 T3	90.3	1
Bealey New 21-25nt				
	AF035637	Ryegrass mosaic virus strain RGMV-AV	99.5	2
	KR061300	Ryegrass mosaic virus isolate Denmark	92.26	9
	AF035818	Ryegrass mosaic virus strain RGMV-AV	94.77	9
	AF035640	Ryegrass mosaic virus strain RGMV-B	97.83	1
	AF191073	Stealth virus 1 clone 3B43,	86.96	1
Bealey New 21-22nt				
	AF035638	Ryegrass mosaic virus strain RGMV-3	99.02	2
	KR061300	Ryegrass mosaic virus isolate Denmark	92.02	10
	AF035818	Ryegrass mosaic virus strain RGMV-AV	95.5	10
	AF035639	Ryegrass mosaic virus strain RGMV-5	96.51	1
	AF035637	Ryegrass mosaic virus strain RGMV-AV	99.5	2
Bealey New 24nt				
	AF035638	Ryegrass mosaic virus strain RGMV-3	99.12	2
	KR061300	Ryegrass mosaic virus isolate Denmark	91.75	8
	AF035818	Ryegrass mosaic virus strain RGMV-AV	94.71	8
	AF035637	Ryegrass mosaic virus strain RGMV-AV	99.49	2
	AF035640	Ryegrass mosaic virus strain RGMV-B	97.83	1
	AF191073	Stealth virus 1 clone 3B43 T3	86.9	1

Table 3.6. BLASTN summary of contigs obtained from LP258 old and new.

Sample	NCBI Accession Number	Virus/Isolate	Average %	Contig Hits #
LP258 Old 21-25nt				
	AF020090	Barley yellow dwarf virus - PAV	98.75	2
	AF235168	Cereal yellow dwarf virus-RPS RPV	88.95	2
	DQ988094	Cereal yellow dwarf virus - RPV	96.73	3
	Y09854	Ryegrass mosaic virus,	91.07	12
	KR061300	Ryegrass mosaic virus isolate Denmark,	91.94	14
	AF035638	Ryegrass mosaic virus strain RGMV-3	98.86	2
	AF035818	Ryegrass mosaic virus strain RGMV-AV,	96.89	14
	AF035637	Ryegrass mosaic virus strain RGMV-AV	99.7	3
	FM865413	Wheat yellow dwarf virus-GPV,	85.58	3
LP258 Old 24nt				
	AF020090	Barley yellow dwarf virus - PAV	98.03	2
	AF235168	Cereal yellow dwarf virus-RPS RPV	87.07	1
	DQ988094	Cereal yellow dwarf virus - RPV isolate	94.9	1
	DQ115529	Cereal yellow dwarf virus - RPV	96.03	1
	KC937023	Cherry leaf roll virus isolate 1978	100	1
	KR061300	Ryegrass mosaic virus isolate Denmark	92.83	12
	AF035818	Ryegrass mosaic virus strain RGMV-AV	97.41	12
	AF035637	Ryegrass mosaic virus strain RGMV-AV	99.7	3
	FM865413	Wheat yellow dwarf virus-GPV	86.83	2
LP258 New 21-22NT				
	Y09854	Ryegrass mosaic virus, complete genome	92.02	14
	KR061300	Ryegrass mosaic virus isolate Denmark	92.51	16
	AF035818	Ryegrass mosaic virus strain RGMV-AV	99.42	18
LP258 New 21-25nt				
	Y09854	Ryegrass mosaic virus	92.04	14
	KR061300	Ryegrass mosaic virus isolate Denmark	92.51	16
LP258 New 24nt				
	AF035818	Ryegrass mosaic virus strain RGMV-AV	99.41	18
	AF020090	Barley yellow dwarf virus - PAV	98.03	2
	L25299	Cereal yellow dwarf virus - RPV	86.06	2
	KC937023	Cherry leaf roll virus isolate 1978	100	1
	Y09854	Ryegrass mosaic virus, complete genome	91.5	10
	KR061300	Ryegrass mosaic virus isolate Denmark	92.83	12
	AF035818	Ryegrass mosaic virus strain RGMV-AV	97.41	12

When subjected to BLASTN analysis, contigs obtained from Bealey old and new exhibited homology to RGMV (>90%) and *Stealth Virus 1*, a dsDNA virus. Contigs obtained from Bealey old and new or young did not exhibit similarity or homology to any other viruses known or suspected to infect ryegrass. In contrast to Bealey, the contigs obtained from LP258 old and new exhibited high degrees of similarity to a range of viruses, such as BYDV-PAV, CYDV-RPS and –RPV, *Wheat yellow dwarf virus* – GPV, which is *BYDV* isolate –GPV, numerous isolates of RGMV, and *Cherry Leafroll Virus (*Family: *Comoviridae*, Genus: *Nepovirus*) a bipartite (+) ssRNA virus not known to infect ryegrass. Overall, a wider range

of viruses were detected in LP258 than in Bealey. However, in both cultivars and across both treatments, a higher proportion of contigs displayed homology to RGMV than to any other virus detected. For example, the average number of contigs with homology to BYDV was ~2-3. In contrast, the average number of contigs with homology to RGMV ranged from 1-18. Lastly, contigs obtained from our samples did not exhibited homology and/or similarity to any *Partitiviruses*, or specifically *Deltapartitiviruses*.

3.6. qRT-PCR:

3.6.1. BYDV: From qRT-PCR, normalized copy number values were obtained overtime for each line, treatment and plot for BYDV. A full account of qRT-PCR data is listed in Appendix 5.

Table 3.7. Summary of BYDV copy number.

LP258 O (25) 15/4/15 - 3252554 18/9/15 - 2276310 17/2/16 - 498449 Ave: 2009104.9	R164 O (26) 15/4/15 - 7834 18/9/15 - Nq 17/2/16 - Nq Ave: 2611.4	R141 N (27) 15/4/15 – Nq 18/9/15 – Nq 17/2/18 - Nq Ave: Nq	Bealey O (28) 15/4/15 – 372649 18/9/15 – 632489 17/2/18 - 614660 Ave: 59932.9	LP256 N (29) 15/4/15 – Nq 18/9/15 – Nq 17/2/16 - 35050 Ave: 11683.6	R164 N (30) 15/4/15 – Nq 18/9/15 – Nq 17/2/16 – Nq Ave: Nq
LP258 N (19) 15/4/15 - 5304 18/9/15 - Nq 17/2/16 – Nq Ave: 1768	BEALEY O (20) 15/4/15 - 68543 18/9/15 - 399041 17/2/16 – 151267 Ave: 206284	LP256 N (21) 15/4/15 - 156858 18/9/15 - 48947 17/2/16 – 402423 Ave: 202743	LP258 O (22) 15/4/15 - 24086 18/9/15 - 4491 17/2/16 – 16633 Ave: 15070	R164 N (23) 15/4/15 - Nq 18/9/15 - Nq 17/2/16 – Nq Ave: Nq	R141 N (24) 15/4/15 - Nq 18/9/15 - Nq 17/2/16 – Nq Ave: Nq
LP258 O (13) 15/4/15 - 520360 18/9/15 - 265298 17/2/16 – Nq Ave: 261886	R141 O (14) 15/4/15 - 732712 18/9/15 - 491683 17/2/16 – Nq Ave: 408131	LP258 N (15) 15/4/15 - Nq 18/9/15 - 3493 17/2/16 – Nq Ave: 1164	BEALEY O (16) 15/4/15 - 795420 18/9/15 - 155167 17/2/16 – Nq Ave: 316862	BEALEY N (17) 15/4/15 - Nq 18/9/15 - Nq 17/2/16 – Nq Ave: Nq	R164 O (18) 15/4/15 - 3716 18/9/15 - 3425 17/2/16 – 43622 Ave: 16921
R141 O (7) 15/4/15 - 93144 18/9/15 - 104584 17/2/16 – 92208 Ave: 96645	LP256 O (8) 15/4/15 - 1518922 18/9/15 - 2035410 17/2/16 – 63739 Ave: 1206024	BEALEY N (9) 15/4/15 - 61465 18/9/15 - 36442 17/2/16 – 2398190 Ave: 832032	R164 O (10) 15/4/15 - Nq 18/9/15 - Nq 17/2/16 – Nq Ave: Nq	R141 N (11) 15/4/15 - Nq 18/9/15 - Nq 17/2/16 – 18385 Ave: 6128	LP258 O (12) 15/4/15 – 432265 18/9/15 - 214171 17/2/16 – 119239 Ave: 255225
LP256 N (1) 15/4/15 - Nq 18/9/15 - Nq 17/2/16 – Nq Ave: Nq	R164 N (2) 15/4/15 - Nq 18/9/15 - Nq 17/2/16 – Nq Ave: Nq	R141 O (3) 15/4/15 - 112858 18/9/15 - 78364 17/2/16 – 32421 Ave: 74548	LP258 N (4) 15/4/15 - 3585 18/9/15 - 2475 17/2/16 – 4666 Ave: 3576	LP256 O (5) 15/4/15 - 760285 18/9/15 - 693202 17/2/16 –6898725 Ave: 714454	BEALEY N (6) 15/4/15 - 77563 18/9/15 - 69147 17/2/16 – Nq Ave: 48903

From the summary data, it can be seen that BYDV transcript levels vary overtime (Table 3.7). Furthermore, within particular lines or cultivars the summary data indicates that BYDV was non-quantifiable at many time points, as represented by "Nq". Due to the presence of non-quantifiable data, copy number values had to be transformed before the data could be

subjected to parametric analysis, such as a repeated measurement ANOVA. Once transformed, a repeated measurement ANOVA was performed.

From the repeated measurement ANOVA of BYDV copy number, it was determined that there is a significant effect between age and viral load (<0.001), cultivar and viral load (<0.001), and a significant interaction between viral load, age and sampling date (<0.021), as summarized in Table 3.8. Full ANOVA output of the qRT-PCR data is presented in Appendix 7. To illustrate the results of the REML ANOVA listed in Table 3.8. the following graphs were generated.

Table 3.8. Summary of results from REML ANOVA for BYDV

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Date	3.43	2	1.72	40	0.193
Age (Old/New)	37.33	1	37.33	20	**<0.001**
Cultivar	32.28	4	8.07	20	**<0.001**
Date of Sampling.Age	8.51	2	4.25	40	**0.021**
Date of Sampling.Cultivar	3.04	8	0.38	40	0.925
Age.Cultivar	6.92	4	1.73	20	0.183
Date of Sampling.Age.Cultivar	5.03	8	0.63	40	0.749

Firstly, Figure 3.5 illustrates significant difference between the mean BYDV viral load of old ryegrass against the mean BYDV load of new ryegrass over time. Essentially, at the time of each sampling event, BYDV viral load of old ryegrass significantly exceeded the viral load of new ryegrass (Figure 3.5).

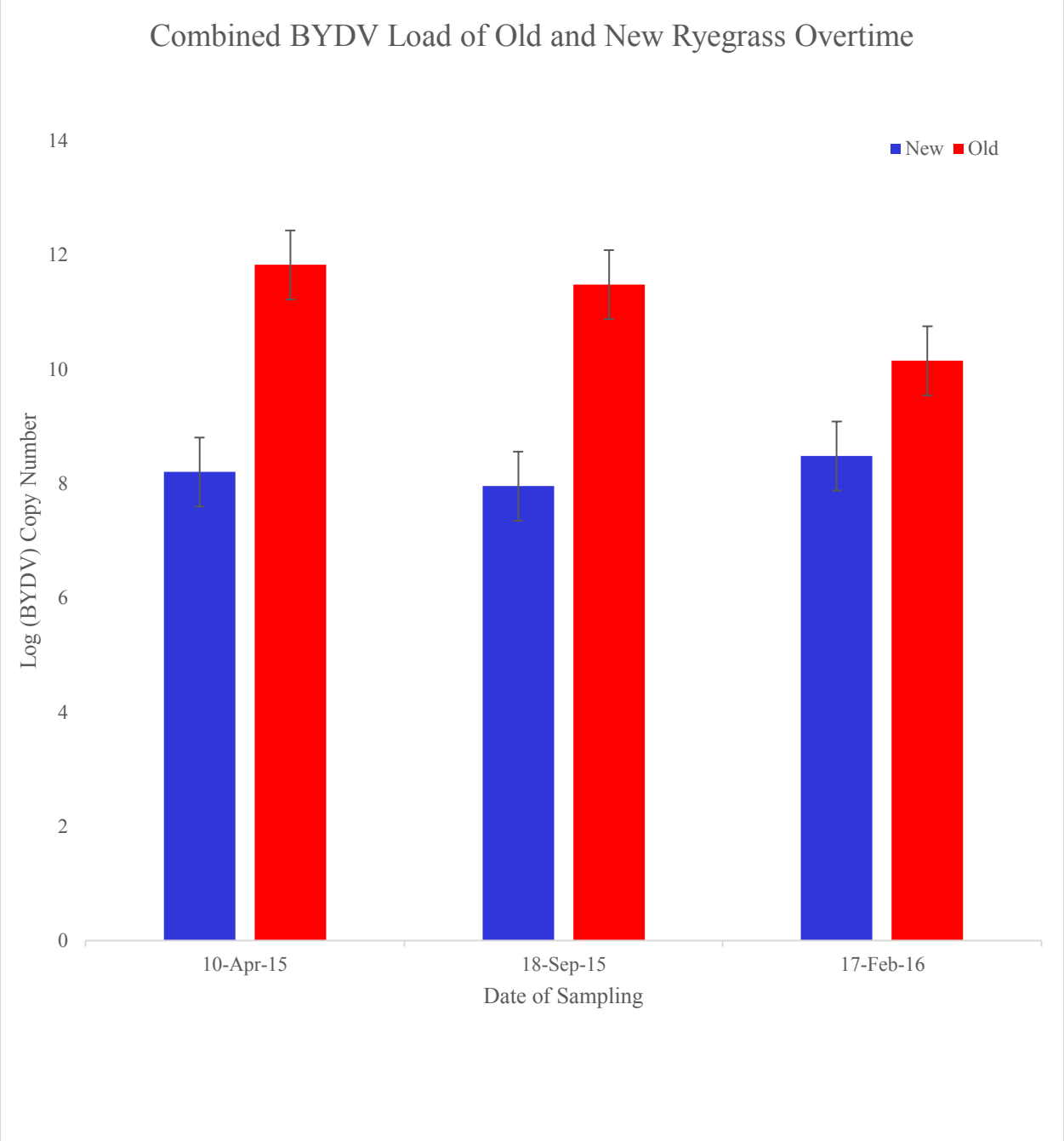


Figure 3.5. Combined BYDV load of old and new ryegrass overtime ($p<0.021$; df, 2; SED, 0.6041).

Secondly, the ANOVA output indicated that a cultivar effect was present or essentially that viral load differs depending upon cultivar, as illustrated by Figure 3.6.

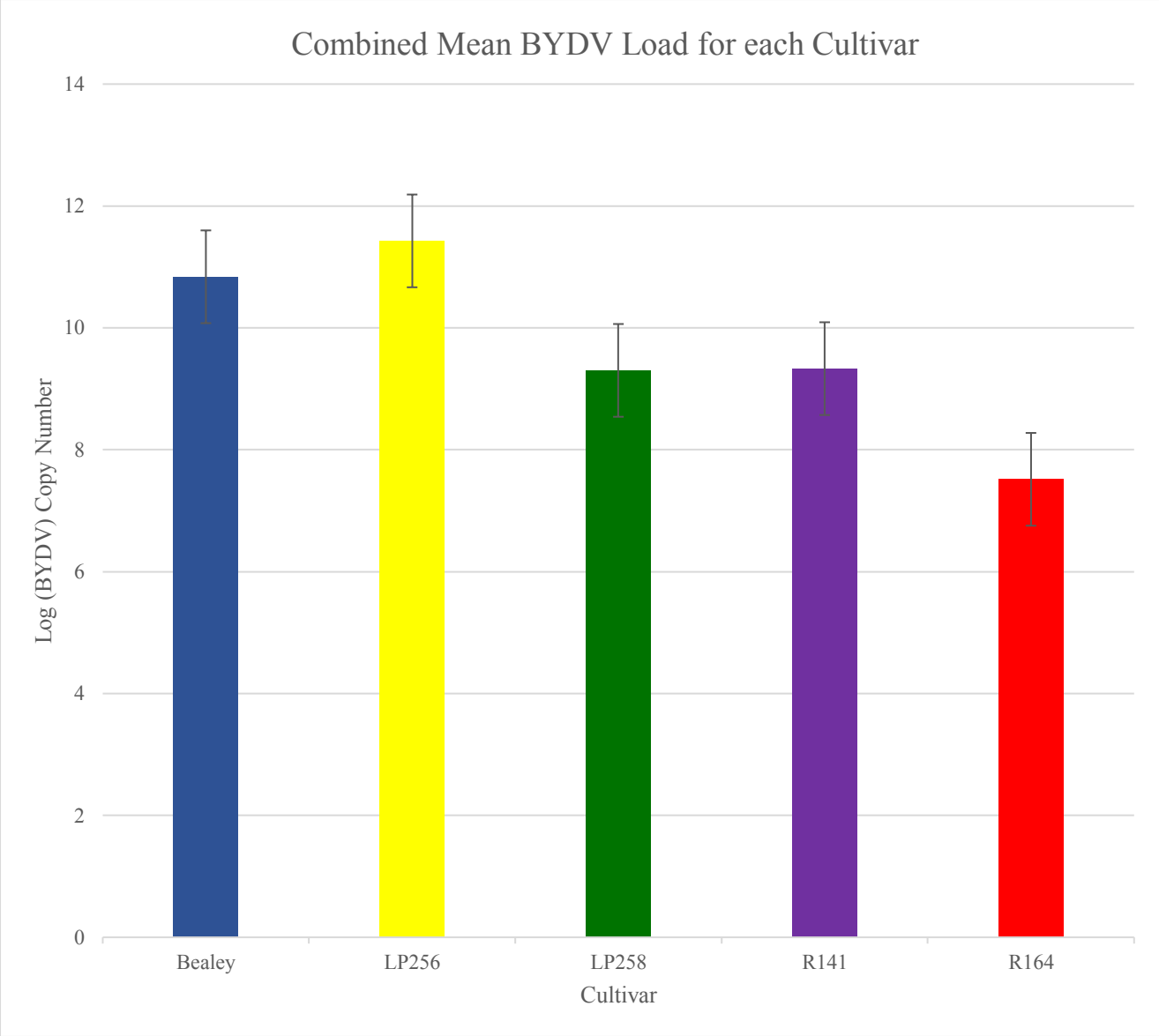


Figure 3.6. Combined mean BYDV load for each cultivar (p<0.001; df, 4; SED, 0.761).

From the REML ANOVA, it was also determined that there is a significant interaction between viral load, age and sampling date, as depicted in Figure 3.7 and 3.8 Specifically, from Figure 3.7 it can be observed that overall the mean viral load of BYDV in old ryegrass is significantly greater than the viral load of new ryegrass.

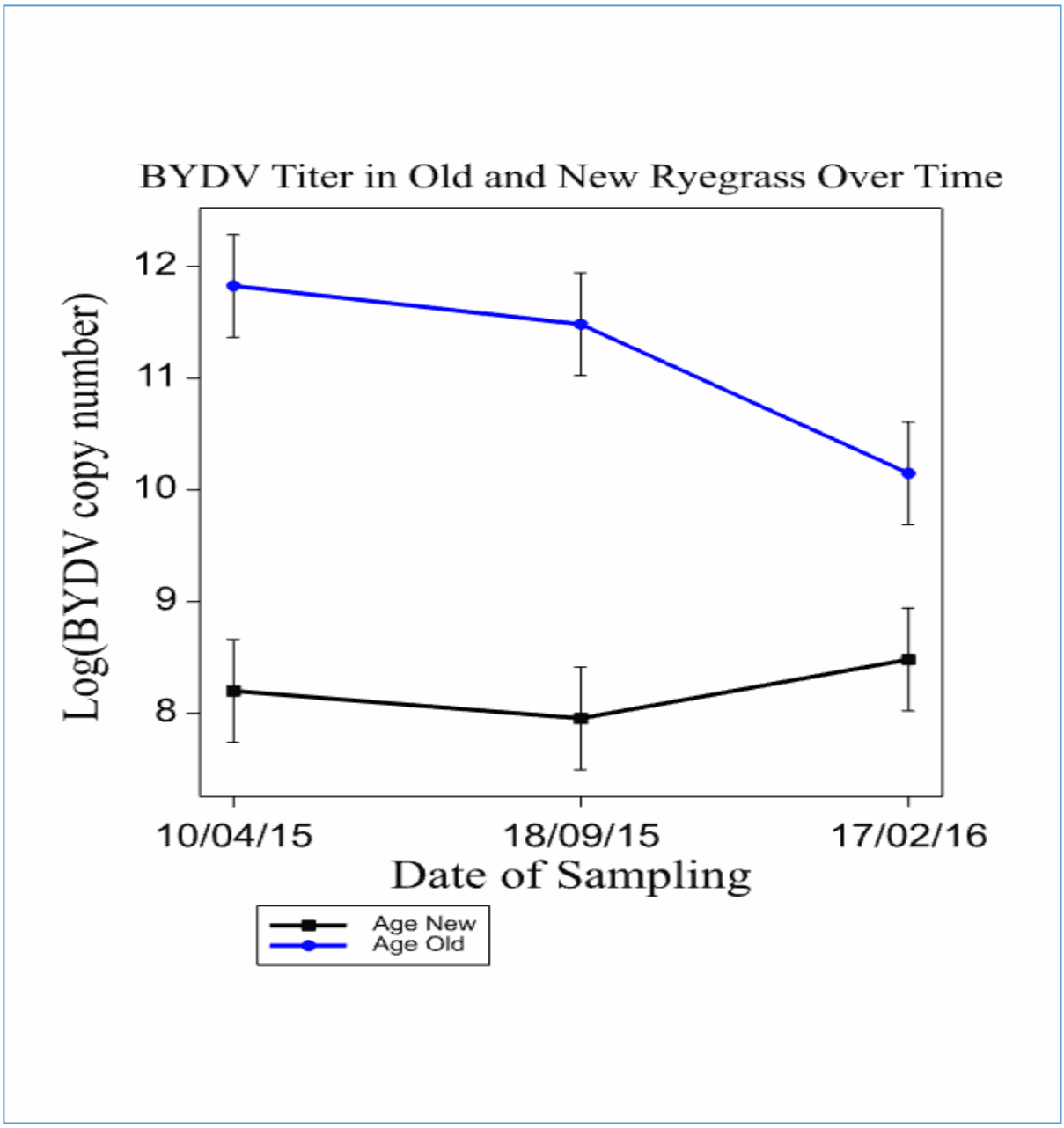


Figure 3.7. Combined mean viral load of BYDV in new and old ryegrass overtime ($p<0.021$; df, 2; SED, 0.3785).

However, when the data is partitioned to reflect viral load over time per treatment for each cultivar, it can be observed that there are differences between cultivars and the treatments (old or new) over time. Specifically, Figure 3.8 demonstrates that whilst the viral load of old ryegrass is generally higher than that of new ryegrass, there are exceptions. For example, the viral load of old and new R164 is not significantly different over time. Furthermore, it can be seen from Figure 3.8 that on the 17/2/16, there appears to be a decline in load across all of the older ryegrass samples, except for R164. Despite this decline, the load of BYDV in old ryegrass remains higher in comparision to the viral load of new ryegrass.

Figure 3.8 also demonstrates that across all young or new ryegrass samples, viral load remains consistently low. Observed differences over time per treatment and/or line can be could potentially indicate the impact of Genotype x Environment x Virus interactions.

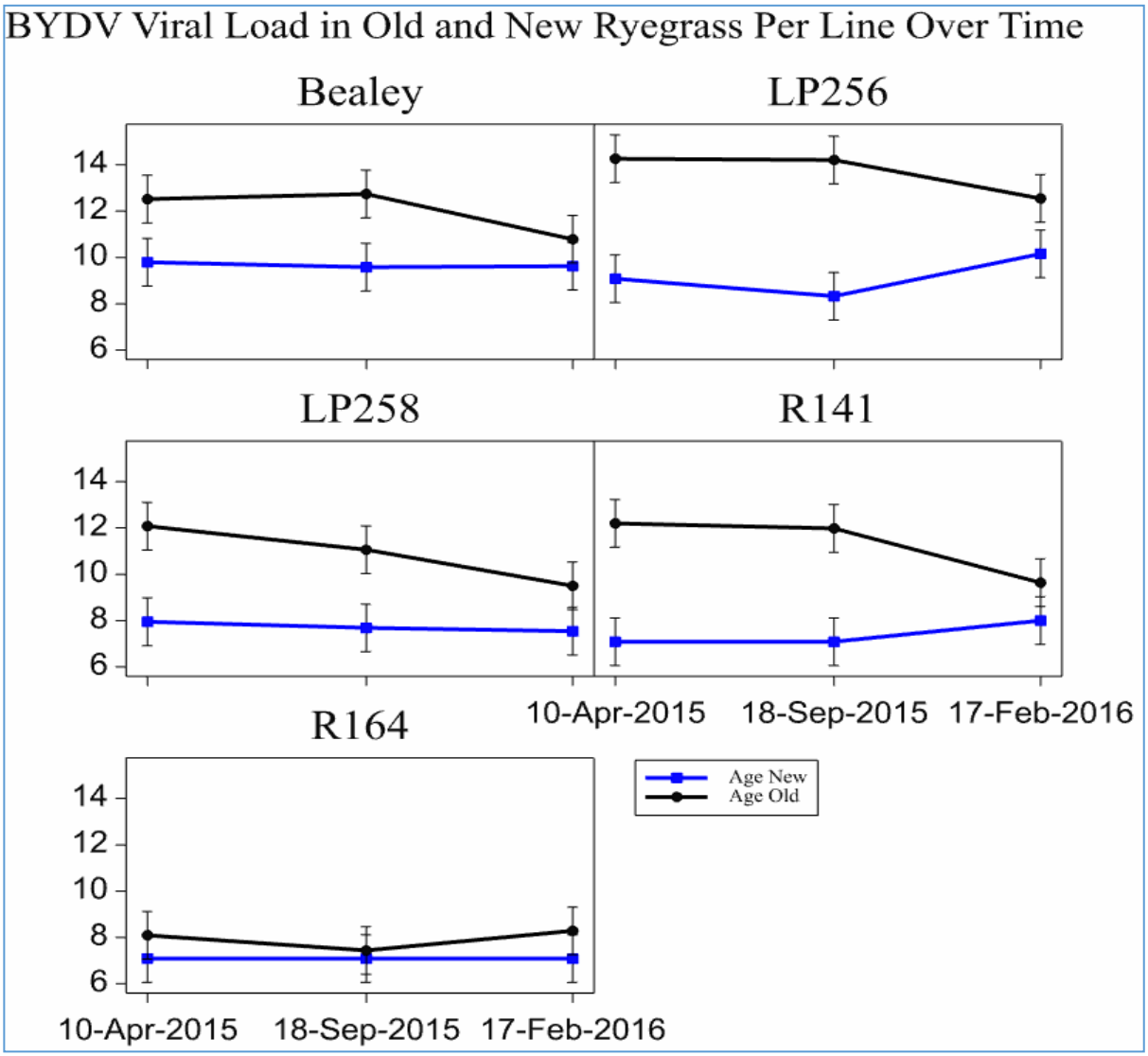


Figure. 3.8. Combined mean viral load of BYDV in old and new ryegrass overtime for each cultivar ($p<0.021$; df, 2; SED, 0.3785).

3.6.2. RGMV: The following copy number values where obtained overtime for each cultivar, treatment and plot for RGMV. Full copy number values are listed in Appendix 5.

Table 3.9. Summary of RGMV copy number

LP258 O (25) 15/4/15 - 3346376 18/9/15 - 2705523 17/2/16 - 2673724 Ave: 2908541	**R164 O (26)** 15/4/15 - 26500974 18/9/15 - 41427408 17/2/16 - 8509008 Ave: 25479130	**R141 N (27)** 15/4/15 – 1417778 18/9/15 – 6708340 17/2/18 - 3074152 Ave: 3733423	**Bealey O (28)** 15/4/15 – 5251139 18/9/15 – 16258324 17/2/18 - 5292799 Ave: 8934087	**LP256 N (29)** 15/4/15 – 1431761 18/9/15 – 2605998 17/2/16 - 2168458 Ave: 2068739	**R164 N (30)** 15/4/15 – 452399 18/9/15 – 5028345 17/2/16 – 738244 Ave: 2072996
LP258 N (19) 15/4/15 - 10357460 18/9/15 - 1189370 17/2/16 – 1596909 Ave: 4381246	**BEALEY O (20)** 15/4/15 - 3430847 18/9/15 - 5624404 17/2/16 – 19122943 Ave: 9392731	**LP256 N (21)** 15/4/15 - 5206819 18/9/15 - 13225453 17/2/16 – 2795115 Ave: 7042462	**LP258 O (22)** 15/4/15 - 22582433 18/9/15 - 15989787 17/2/16 – 6936595 Ave: 15169605	**R164 N (23)** 15/4/15 - 2238203 18/9/15 - 16032948 17/2/16 – 3039142 Ave: 7103431	**R141 N (24)** 15/4/15 - 6862123 18/9/15 - 9227454 17/2/16 – 2361596 Ave: 6150391
LP258 O (13) 15/4/15 - 7106905 18/9/15 - 868353 17/2/16 – 6360276 Ave: 4778511	**R141 O (14)** 15/4/15 - 14563613 18/9/15 - 5355856 17/2/16 – 16806105 Ave: 12241858	**LP258 N (15)** 15/4/15 - 3993995 18/9/15 - 13081137 17/2/16 – 2152190 Ave: 6409107	**BEALEY O (16)** 15/4/15 - 7906700 18/9/15 - 7009957 17/2/16 – 9208369 Ave: 8041675	**BEALEY N (17)** 15/4/15 - 164767 18/9/15 - 832735 17/2/16 – 215125 Ave: 404209	**R164 O (18)** 15/4/15 - 12272232 18/9/15 - 26147267 17/2/16 – 24128414 Ave: 20849304
R141 O (7) 15/4/15 - 8734888 18/9/15 - 22873617 17/2/16 – 16073133 Ave: 15893879	**LP256 O (8)** 15/4/15 - 4572968 18/9/15 - 13700905 17/2/16 – 13477839 Ave: 10583904	**BEALEY N (9)** 15/4/15 - 1187995 18/9/15 - 55302210 17/2/16 – 1821245 Ave: 19437151	**R164 O (10)** 15/4/15 - 14667704 18/9/15 - 3654636 17/2/16 – 13271335 Ave: 10531225	**R141 N (11)** 15/4/15 - 2155230 18/9/15 - 15837631 17/2/16 – 2829083 Ave: 6940648	**LP258 O (12)** 15/4/15 – 6156639 18/9/15 - 8045440 17/2/16 – 6679290 Ave: 6960456
LP256 N (1) 15/4/15 - 238340 18/9/15 - 19855867 17/2/16 – 442277 Ave: 888828	**R164 N (2)** 15/4/15 - 930141 18/9/15 - 758095 17/2/16 –2140895 Ave: 1276377	**R141 O (3)** 15/4/15 - 8253006 18/9/15 - 22892401 17/2/16 – 11578069 Ave: 14241159	**LP258 N (4)** 15/4/15 - 2180351 18/9/15 - 4129921 17/2/16 – 3937453 Ave: 3415909	**LP256 O (5)** 15/4/15 - 4763618 18/9/15 - 9894524 17/2/16 –8794158 Ave: 7817433	**BEALEY N (6)** 15/4/15 - 1105744 18/9/15 - 3578141 17/2/16 – 1796622 Ave: 2160169

In contrast to BYDV, the summary data obtained from qRT-PCR analysis indicates that RGMV is present within all samples at relatively persistent or stable transcript levels that appear to increase overtime (Table 3.9). Copy number values were transformed and subjected to a repeated measurement ANOVA.

From the REML ANOVA of RGMV data, it was determined that there is a significant effect between date and viral load (<0.003), between age and viral load (<0.001), and a significant interaction between date of sampling, viral load and age (<0.006), as summarized in Table 3.10. Full ANOVA output is documented in Appendix 7.

Table 3.10. Summary of results from REML ANOVA for RGMV

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Date	13.31	2	6.65	40	**0.003**
Age	25.75	1	25.75	20	**<0.001**
Cultivar	5.28	4	1.32	20	0.297
Date of sampling.Age	11.74	2	5.87	40	**0.006**
Date of sampling.Cultivar	9.63	8	1.2	40	0.321
Age.Cultivar	3.61	4	0.9	20	0.482
Date.Age.Cultivar	2.51	8	0.31	40	0.956

The REML ANOVA output was used to generated the following graphs. Firstly, Figure 3.9 illustrates the mean RGMV viral load of old ryegrass against the mean RGMV viral load of new ryegrass overtime. From Figure 3.9 it be can be observed that the viral load of older ryegrass significantly exceeds the viral load of new ryegrass.

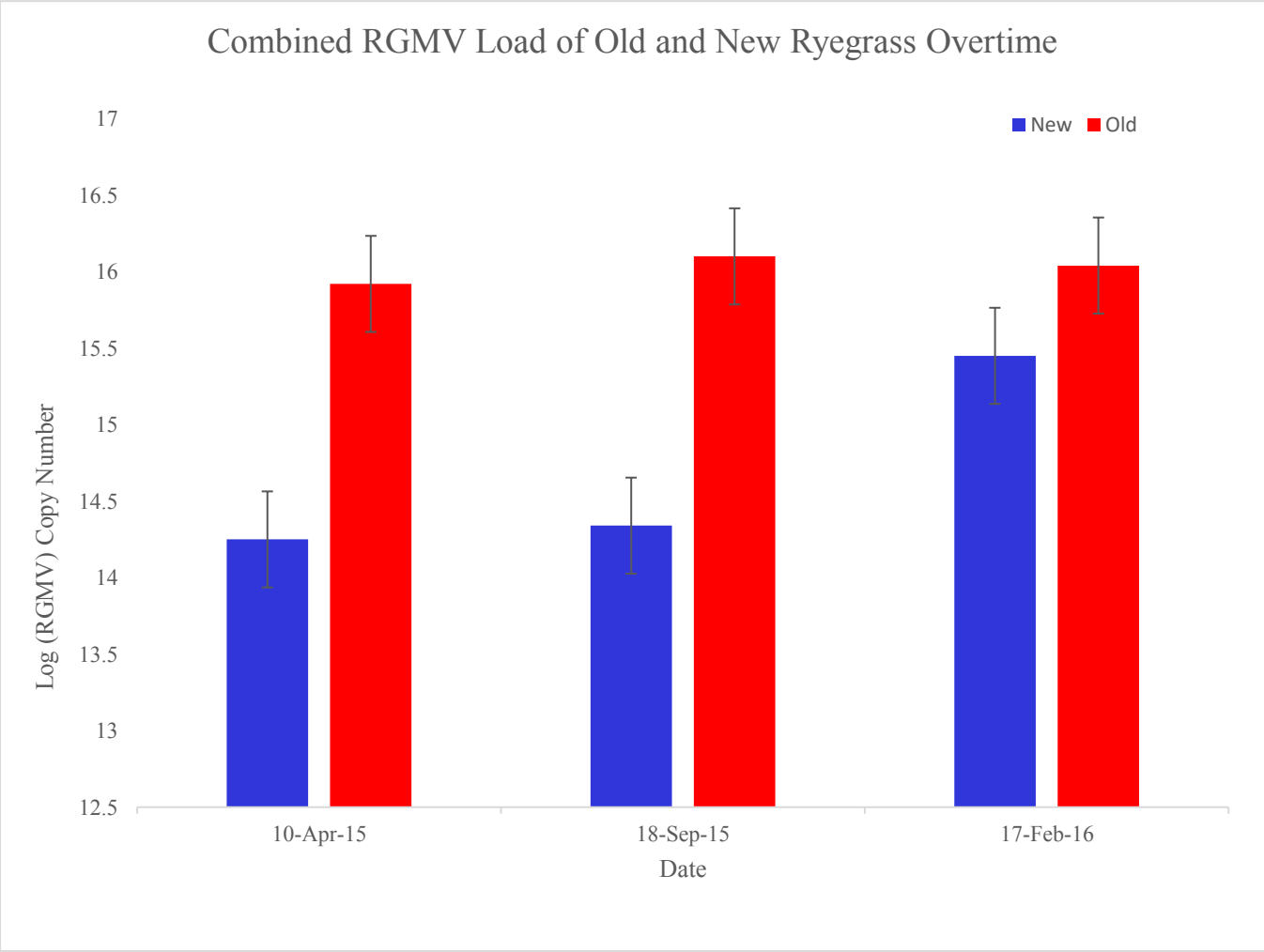


Figure 3.9. Combined mean of viral load for old and new ryegrass overtime (p<0.006; df, 2; SED 0.3140).

Secondly, from analysis of RGMV viral load within old and new ryegrass overtime, it can be seen that the RGMV viral load of old ryegrass is significantly greater than the RGMV viral load of new ryegrass (Figure 3.10). From Figure 3.10 it can also be observed that the viral load or concentration of RGMV in new ryegrass increases overtime, whilst the load of RGMV in old ryegrass appears to plateau. Despite this, the viral load of old ryegrass remains significantly higher than the viral load of new ryegrass.

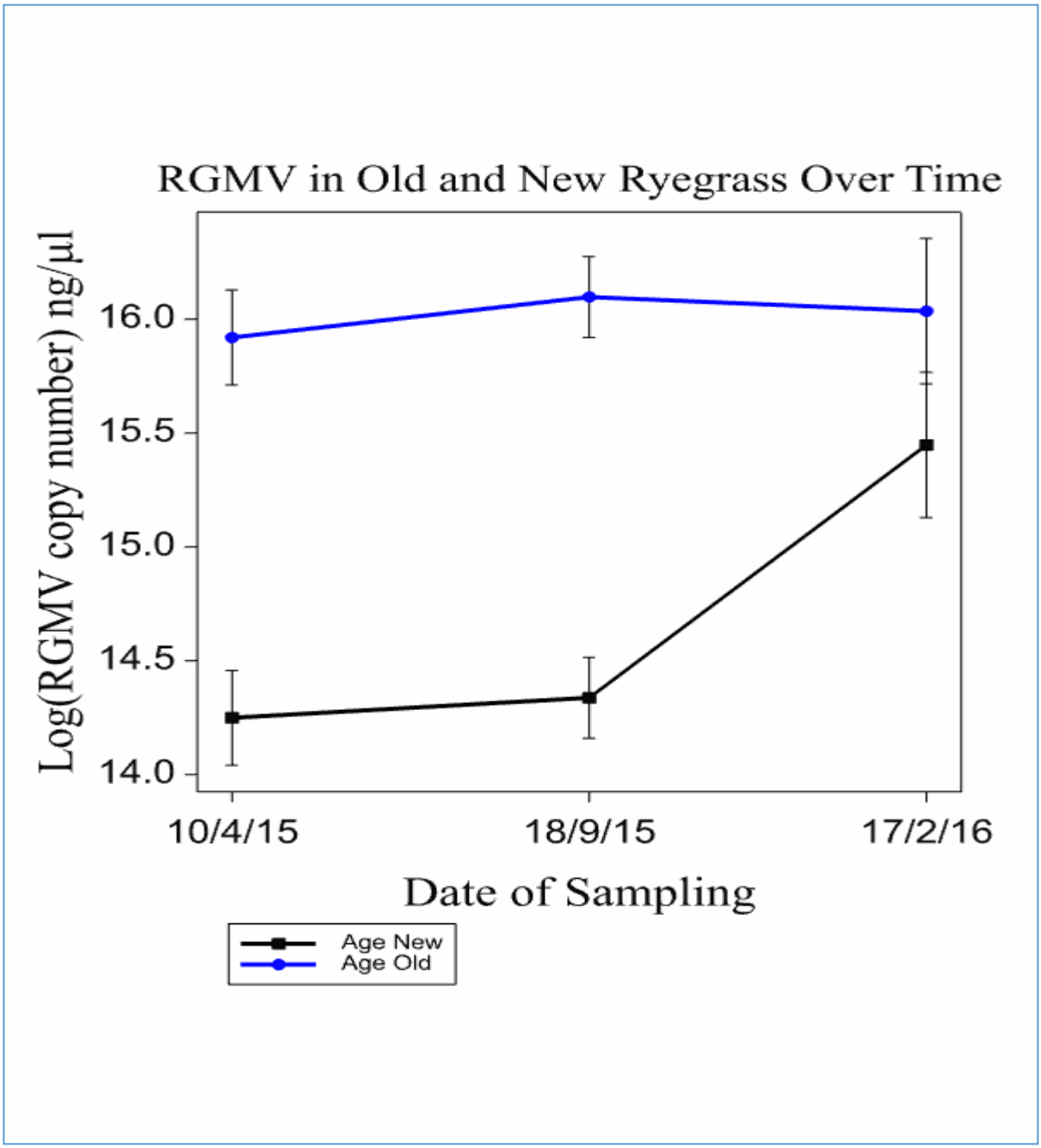


Figure. 3.10. Combined mean viral load of RGMV in new and old ryegrass overtime ($p<0.006$; df, 2; SED 0.3140)

Figure 3.11 depicts the mean viral load for each cultivar overtime. From this figure, it can be observed the that viral load of old ryegrass consistently exceeds the viral load of new or young ryegrass within each cultivar overtime. Figure 3.11 also illustrates that the load or titre of RGMV within new ryegrass for each cultivar appears to be increase over time. Lastly, observed differences in viral load per treatment and/or cultivar over time, may indicate the presence of Genotype x Virus x Environment interactions (Figure 3.11).

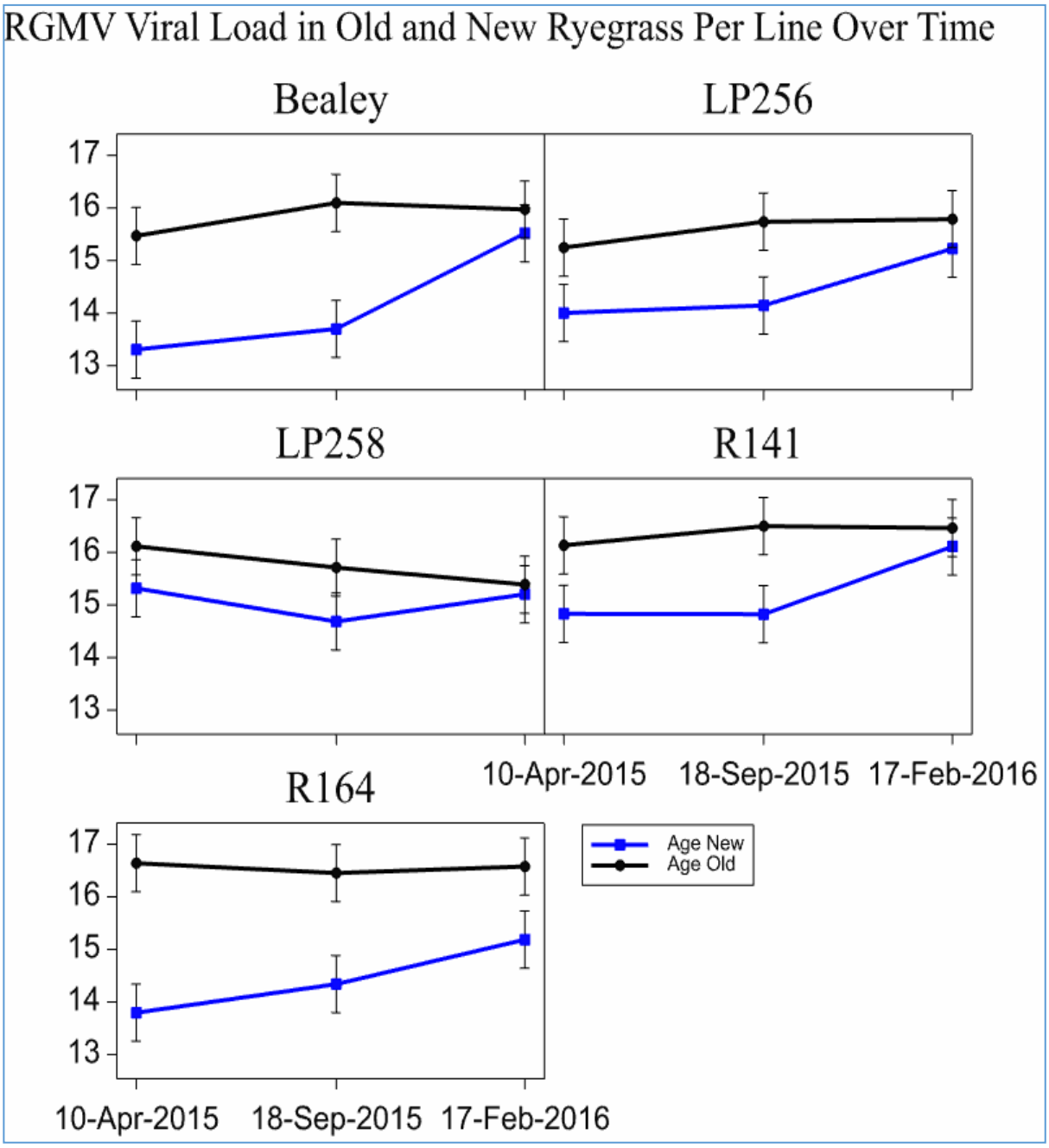


Figure 3.11. Combined mean viral load of RGMV in old and new ryegrass overtime for each cultivar ($p<0.006$; df, 2; SED 0.3140).

3.7. Results Summary:

Yield Deficit: The results of objective 1 demonstrate that overall the yield of young ryegrass is significantly greater than the yield of old ryegrass (Figure 3.1). Depending upon the line, the yield of young or new ryegrass was 4-29% greater than the yield of old ryegrass. The yield between young and old was significantly different for all lines, except LP258. These results indicate that GxE interactions are present, as observed by differences in yield between each of the respective cultivars examined (Table 3.2 and Figures 3.2 and 3.3). Furthermore, it can be observed from the results that yield or ryegrass dry matter production declines over time (Table 3.2 and Figure 3.3). Lastly, Figure 3.4 illustrates the difference between old and new ryegrass material with regards to quality. Specifically, this image demonstrates that old ryegrass was less vigorous than young ryegrass.

Multiple Viruses: The presence of multiple viruses or the co-infection of ryegrass samples by a range of viruses was confirmed by a range of methods. The results obtained from ELISA, siRNA analysis and qRT-PCR demonstrate that multiple viruses were present within the ryegrass samples examined (Tables 3.3, 3.4, 3.5, 3.6. 3.7 and 3.9).

Ryegrass Age & Viral Load: The results of qRT-PCR, indicate that overall the viral load of 10-year-old ryegrass exceeded that of new ryegrass, as expected (Tables 3.7 and 3.9., and Figures 3.5, 3.7, 3.9 and 3.10). Additionally, from the results RGMV infection appears to be prevalent across all cultivars overtime with transcript levels that are consistently higher than BYDV (Tables 3.7 and 3.9, and Figures 3.7 and 3.10). Furthermore, RGMV's titre appears to increase consistently overtime within each sample examined, whilst BYDV titre appears to fluctuate and differ within each cultivar over time (Table 3.8 and Figures 3.6, 3.7, 3.8 and 3.11). Overall, the results indicate that a potential correlation or link may exist between decreased yield and persistence in ryegrass and viral infection. Specifically, it is tenable that RGMV and/or BYDV and RGMV infection combined, could have contributed to the observed decline in yield and/or persistence of older ryegrass material, as demonstrated by Figures 3.1, 3.3, in which yield or yield stability (persistence) declines as there is a concomitant increase in viral load over time (Figures 3.8 and 3.11). However, the results of this project indicate that a range of interactions are likely to influence the overall impact and outcome of viral pathogenesis

Chapter Four

Discussion:

Increasing pasture performance is a primary objective within the agricultural/pastoral sector in N.Z. However, as previously mentioned, if one wishes to enhance pasture productivity, one must appreciate and understand the extent to which environmental factors, such as viruses, can modify or reduce pasture yield and persistence *in situ* overtime. Once the magnitude of viral infection is understood, steps can be developed to mitigate losses potentially attributable to this particular factor. The purpose of this research was to elucidate the impact of multiple viruses and viral load upon the yield and performance of ryegrass overtime. The methods used during this project enabled the following trends to be observed.

4.1. Ryegrass & Viral Load: Firstly, qRT-PCR data confirmed that overall, the viral load of 10-year-old ryegrass material exceeded the viral load of new ryegrass material examined, as expected (Figures 3.5, 3.7, 3.9, 3.10). The notable difference in viral load detected between old and new ryegrass is consistent with the findings of previous research conducted within N.Z. and other agricultural sites, in which it was observed that the incidence or frequency of BYDV and RGMV viral infection was generally higher in pastures greater than 4 years old (Latch, 1977; Webster et al.,1996; Jones, 2013; Guy, 2014).

The results of this project also indicate that whilst viral load is substantially greater in older ryegrass material, the infection load or titre of both RGMV and BYDV differed between cultivars over time (Figures 3.6, 3.8 and 3.11), as anticipated. This observation or trend is consistent with the findings of previous research, in which the impact of BYDV and/or RGMV differs depending upon host genotype (A'Brook & Heard, 1975; Wilkins & Catherall, 1977; Catherall, 1987; Catherall & Parry, 1987; Eagling et al., 1989; Guy, 1993; Webster et al., 1999; Remold, 2002; Lehtonen et al., 2006). The observed variation between cultivars could potentially be attributable to genotypic and/or epigenetic variation between ryegrass lines, which in turn could alter the effectiveness of stress responses or host defense mechanisms. In the case of plant-pathogen interactions, the evolutionary arms race between plants and viruses exerts a strong directional pressure on both species to develop mechanisms that enhance or promote their own survival (Buchanan et al., 2000; Garcia-Arenal & McDonald, 2003).

This directional pressure can lead to development of short and/or long term transgenerational phenotypic variation within the population, which can inevitably alter the response of the host to the pathogen (resistance, tolerance and acclimation), and therefore the overall outcome of pathogen infection itself (Agrios, 2005; Kasaga & Gijzen, 2013; Hull, 2014). Recent research indicates that phenotypic variation can develop rapidly within a population due to epigenetic mechanisms (Stern et al., 2007; Bokyko & Kovalchuk, 2011; Feil & Fraga, 2012; Gutzat & Schied, 2012; Ou et al., 2012; Zhang et al., 2013; Crisp et al., 2016). Specifically, it has been observed that environmental stresses can modify cytosine methylation patterns (loss or addition of methyl groups) and/or activate histone modification or rearrangements (de-acetylation/acetylation) (Luekens & Zhan, 2007; Bokyko & Kovalchuk, 2011; Feil & Fraga, 2012; Ou et al., 2012; Migicovsky & Kovalchuk, 2013; Zhang et al., 2013; Crisp et al., 2016). Methylation patterns and histone modifications regulates access to, and thus the transcription of genetic information situated within dense heterochromatin (Fujimoto, 2012). Transcription within these regions is generally repressed (King et al, 2010; Pal & Taylor, 2016; Gutzat & Scheid, 2012; Crisp et al., 2016). However, environmental stress, such as drought, pathogen attack and heavy metal exposure, can modulate or alter the expression and/or repression of repeat sequences situated within these regions by altering patterns of methylation (Lukens & Zhan, 2007; Boyko & Kovalchuk, 2011; Feil & Fraga, 2012; Gutzat & Schied, 2012; Ou et al., 2012; Zhang et al., 2013; Crisp et al., 2016). Furthermore, methylation of sequences within heterochromatin can increase the rate of transposition, specifically the movement, insertion and expression of transposable elements (Acquaah, 2007; Pierce, 2010; Mirouza & Packowski, 2011; Federoff, 2012) Transposable elements can alter existent DNA sequences, generating novel genotypic (phenotypic) variation (King et al., 2010; Pierce, 2010; Federoff, 2012). Essentially, epigenetic mechanisms can increase and/or repress transcription of a range of sequences within the genome, which in turn, can generate novel transient, and reversible, but in some documented cases, heritable genetic variation (Luekens & Zhan, 2007; Bokyko & Kovalchuk, 2011; Feil & Fraga, 2012; Ou et al., 2012; Migicovsky & Kovalchuk, 2013; Zhang et al., 2013; Crisp et al., 2016). For example, in tomato plants (*Solanum lycopersicum* L.) infected with TMV (*Tobacco mosaic virus*), demethylation occurred at loci associated with defense responses (Bokyko & Kovalchuk, 2011; Migicovsky & Kovalchuk, 2013). Demethylation at these loci reportedly delayed the on-set of symptoms (Bokyko & Kovalchuk, 2011; Migicovsky & Kovalchuk, 2013). Furthermore, in rice (*Oryza sativa* L.), exposure to heavy metal stress caused hypomethylation at CHG sites, which was in turn, associated with enhanced tolerance (Ou et al., 2012). Epigenetic mechanisms such as those

described could enable organisms to mount immediate responses to a range of environmental stresses that confer short-term acclimation and/or long term tolerance or resistance/adaptation by modulating the transcriptome (Luekens & Zhan, 2007; Boyko & Kovalchuk, 2011; Feil & Fraga, 2012; Gutzat & Schied, 2012; Ou et al., 2012; Zhang et al., 2013; Crisp et al., 2016). Essentially, alteration of methylation patterns and/or histone modification could contribute to phenotypic diversification between genetically related individuals, and potentially, transgenerational or long term adaptive responses to stress (Luekens & Zhan, 2007; King et al, 2010; Bokyko & Kovalchuk, 2011; Feil & Fraga, 2012; Gutzat & Schied, 2012; Ou et al., 2012; Kasaga & Gijzen, 2013; Zhang et al., 2013; Crisp et al., 2016). As such, it is possible, that the observed differences in viral load over time between cultivars could be a reflection of epigenetically derived or mediated phenotypic variation, which at the molecular level, could have potentially enhanced defense mechanisms altering the accumulation of viruses' or viral load overtime within the material examined (Bokyko & Kovalchuk, 2011; Migicovsky & Kovalchuk, 2013).

4.2. Ryegrass Yield and Persistence: In addition to demonstrating that viral load differs between old and new ryegrass and between cultivars over time, the results of this project also demonstrate that on average the yield of new ryegrass exceeded the yield of old ryegrass, as expected (Figure 3.1 and 3.2). However, differences in yield between old and new varied depending upon cultivar (Table 3.2 and Figure 3.2). For example, between R141 old and new, there was a 34% difference in yield. In contrast, between LP258 old and new there was a ~4% difference in yield (Table 3.2, and Figure 3.2). Furthermore, from the agronomic data it was observed that there is a downward trend in dry matter production overtime for each cultivar examined (Figure 3.3). At the outset of this research, it was anticipated that the yield and persistence of new ryegrass material would be greater than the yield and persistence of old ryegrass, and the results obtained during this project support this. However, there is no data or research to compare these results to, as in contrast to previous investigations, this project had access to a unique resource, namely 10-year-old ryegrass material and the corresponding seed. This resource enabled the yield and persistence of old and new ryegrass across 5 different lines to be compared overtime for the first time. The differences observed with regards to yield between old and new are typical of losses associated within single BYDV or RGMV infections (A'Brook & Heard, 1975; Wilkins & Hide, 1976; Wilkins & Catherall 1977; Latch, 1980; Eagling et al., 1989; Eagling et al., 1992; Webster et al., 1999;

Jones, 2013). However, it is plausible that the observed yield differences between old and new may reflect the impact of environmental variation and/or age-related factors.

4.3. Co-infection of Ryegrass: The results of this project also confirmed the presence of multiple viruses within the ryegrass material examined (Tables 3.3, 3.4, 3.5, 3.6. 3.7 and 3.9). Specifically, RGMV and BYDV infection occurs simultaneously within the ryegrass material examined, as anticipated. The co-infection of ryegrass by multiple viruses is consistent with the findings of other research, which indicate that the co-infection of agricultural plants by multiple viruses occurs frequently within natural pathosystems, (Carfrune et al., 2006; Caracuel et al., 2012; Syller, 2012; Mascia & Gallitelli, 2016). The presence of multiple homologous or heterologous viruses within a given host has the potential to influence and/or alter host-virus interactions, and therefore the overall outcome of viral infection (Syller, 2012; Mascia & Gallitelli, 2016; Syller & Grupa, 2016). It has been reported that virus-virus interactions are predominately synergistic, during which the interaction has a facilitative effect upon both or at least one of the viral partners (Pruss et al., 1997; Caracuel et al., 2012; Syller, 2012; Mascia & Gallitelli, 2016). Synergistic or facilitative interactions are reportedly characterized by the increased replication and accumulation of one or more of the viral partners within the host, as well as the manifestation or induction of symptoms more severe than that associated with a single virus (Mascia & Gallitelli, 2016; Syller & Grupa, 2016). Numerous synergistic interactions involving members of the potyvirus family, such as RGMV, have been described (Pruss, et al., 1997; Syller, 2012; Syller & Grupa, 2016). Generally, mixed infections involving a potyvirus and a heterologous or unrelated virus, results in a dramatic increase in symptom severity, and the accumulation of the co-infecting virus(es) (Pruss et al., 1997; Syller, 2012; Mascia & Gallitelli, 2016). Furthermore, the overall outcome of pathogenesis or the impact of viruses operating in a synergistic manner has a general tendency to exacerbate or increase the impact of virus infection, such as exacerbating yield losses, as has been observed in other monocotyledonous crop species (Eagling et al., 1992; Carfrune et al, 2006; Guy, 2014). For example, garlic (*Allium sativum* L.) plants infected with multiple viruses or a "virus-complex" experienced significantly greater yield losses (up to 61%) in comparison to garlic plants infected with a single virus (up to 25% reduction) (Carfrune et al, 2006). In accordance with previous observations it was anticipated that the combined presence of RGMV and BYDV within ryegrass would exacerbate the impact of viral infection, as exemplified by concurrent increases in the load or titre of both

viral pathogens, as well as yield losses in excess of those typically associated with a single virus (i.e. >20%). (A'Brook & Heard, 1975; Wilkins & Hide, 1976; Latch, 1980; Eagling et al., 1992; Wilkins & Catherall, 1997; Webster et al., 1999; Carfrune et al, 2006; Guy, 2014; Mascia & Gallitelli, 2016; Syller & Grupa, 2016). In contrast to previous findings, neither the impact nor titre of RGMV and/or BYDV appeared to be exacerbated or enhanced irrespective of concurrent infection of the host. Specifically, yield losses between old and new did not exceed levels typically associated with a single virus infection and RGMV's transcript levels were consistently higher than that of BYDV across all samples and overtime (Tables 3.7 and 3.9, and Figures 3.7 and 3.10). Unexpectedly, the results of this project indicate that RGMV and BYDV may interact antagonistically, rather than synergistically. An antagonistic interaction between viruses within a host is characterized by the decreased replication and/or inhibition of one virus by another (Power, 1996; Syller, 2012; Syller & Grupa., 2014; Mascia & Gallitelli, 2016).

However, it remains equally plausible that RGMV and BYDV interact synergistically, but that other factors may have altered the impact and accumulation of these viruses within ryegrass during this project. For example, variation in temperature may have influenced the abundance or density of vectors within the experimental site, altering the rate of transmission between prospective hosts, and therefore the transmission of viral inoculum *in situ* (Power, 1996; Finlay and Luck, 2011; Neofytou et al., 2016). Specifically, in regards to RGMV and BYDV, it is possible that the vector of RGMV, the eriophyid mite *Abacarus hystrix* (Nalepa), was more prevalent within the surrounding environment, than the vector of BYDV, enabling RGMV loads to accumulate and reach higher levels than BYDV throughout the duration of the project (Power, 1996; Syller, 2012; Lacriox et al., 2014). Additionally, greater abundance of *Abacarus hystrix* could have facilitated early infection of ryegrass with RGMV, enabling RGMV to attain higher titre within the ryegrass material examined prior to the arrival of BYDV inoculum. It has previously been suggested that the spatiotemporal order of arrival of viruses on the host could alter the outcome of mixed infections (Mascia & Gallitelli, 2016).

Ultimately, this project demonstrates that there is an appreciable difference between old and new ryegrass with regards to viral load, yield and performance overtime, and whilst a tenable link between ryegrass yield deficit and increasing viral load can be observed from the results, a definitive correlation between viral load and ryegrass yield decline was not be established. Specifically, this research demonstrates that the interaction between viruses and ryegrass is

complex, and as previously discussed, a range of factors can modify this interaction. Consequently, delineating the actual impact of viruses, such as BYDV and RGMV, upon ryegrass remains challenging. As such, it is remains plausible that the observed decline in ryegrass yield and persistence, or simply, differences in the performance of old and young, could potentially be attributable to other factors, such as epigenetics and ageing, and/or alternative environmental factors, pathogens or pests.

4.4. Alternative factors:

Whilst epigenetic mechanisms can lead to the emergence of novel phenotypic variation in response to environmental stimuli, they can also contribute to or regulate the process of cellular senescence or decay, associated with aging (Hopkin & Huner, 2004; Taiz & Zeiger, 2006; Ay et al., 2014; Pal & Taylor, 2016). Senescence or aging is a genetically encoded time-dependent process that results in the decay or decline of cellular and physiological function (Hopkin & Huner, 2004; Taiz & Zeiger, 2006; Pal & Taylor, 2016). Senescence or aging can occur in response to development and/or environmental cues (Taiz & Zeiger, 2006). In perennial species, such as ryegrass, senescence occurs at the end of the growing season in response to environmental changes, such as photoperiod and temperature (Hopkin & Huner, 2004; Taiz & Zeiger, 2006). The process of senescence can enhance susceptibility to abiotic and/or biotic environmental stresses (Taiz & Zeiger, 2006; Pal & Taylor, 2016).

Cellular and physiological decline is regulated by the epigenome and endo- and/or exogenous factors. As stated before, epigenetic mechanisms are responsible for maintaining genome stability and for coordinating gene expression through the life span of an organism (Feil & Fraga, 2012; Pal & Taylor, 2016). However, in response to particular environmental and/or intrinsic cues, these mechanisms can cause genome instability resulting in transcriptional drift (Feil & Fraga, 2012; Ay et al., 2014; Neofytou et al., 2016; Pal & Taylor, 2016). Specifically, nucleotide sequences or protein-coding genes situated within dense heterochromatin that are normally repressed or transcriptionally silent can undergo hypomethylation or demethylation becoming transcriptionally active (Feil & Fraga, 2012; Pal & Taylor, 2016). Furthermore, hypomethylation or demethylation within heterochromatin can increase transposition and the emergence or accumulation of deleterious DNA mutations overtime (Feil & Fraga, 2012; Ay et al., 2014; Crisp et al., 2016; Neofytou et al., 2016; Pal & Taylor, 2016). For example, in plants, senescence is regulated by increased expression of senescence associated genes

(SAGs) and a concomitant repression of senescence down-regulated genes (SDGs), derepression of heterochromatin, alteration of histones, increased transposition and the emergence of mutations, in response to environmental stimuli (Hopkin & Huner, 2004; Taiz & Zeiger, 2006; Ay et al., 2014). Furthermore, during the life span of an individual organism, cells can undergo a variety of changes, and molecular damage attributable to environmental, genetic and epigenetic factors (Taiz & Zeiger, 2006; Ay et al., 2014; Pal & Taylor, 2016). As such, molecular damages or alterations within developmental and/or stress responsive pathways can accumulate within the genome and/or epigenome over time (Taiz & Zeiger, 2006; Ay et al., 2014; Pal & Taylor, 2016). Senescence or cellular decay, and the accumulation of molecular damage, is associated with a decline in function, and therefore increased susceptibility to environmental perturbations or pathogens, such as viruses (Pal & Taylor, 2016). Therefore, it is possible that observable differences between old and young ryegrass are the consequence of transcriptional drift and/or cellular damage that accumulated overtime within old ryegrass, reducing the growth and capacity of old ryegrass and its capacity to effectively tolerate stresses such as viral infection. However, viruses are not the only stress factor that ryegrass is likely to have encountered.

During this project, abiotic stresses, such as drought, temperature extremes and poor soil nutrients, in combination with cellular decay (and potentially viruses), may have exacerbated the effects of stress, reducing the yield and persistence of old ryegrass (Stewart et al., 2014). Furthermore, as the ryegrass used during this project lacked endophyte, it is possible that pests such as argentine stem weevil (*Listronotus bonariensis*), native grass grab *(Costelytra zealandica*) or nematodes contributed to the observed decline of old ryegrass due to heightened susceptibility (Stewart et al., 2014). However, insect damage upon the ryegrass plots was not observed during this research, as such it remains possible that other stress factors or pathogens could have reduced the yield and persistence of old ryegrass (Stewart et al., 2014). For example, fungal pathogens such as crown rust *(Puccinia coronata*) or stem rust *(Puccinia graminis*) may have contributed to a decline in yield and/or persistence (Stewart & Hayes, 2011; Stewart et al., 2014). However, fungal diseases or rusts on ryegrass are easily discernable, and as such, ryegrass samples were routinely checked for their presence, but no fungal pathogens were observed or detected on the ryegrass plots throughout the duration of this project, therefore it's unlikely that insects and/or fungal pathogens contributed to the reduced the yield and/or persistence of older ryegrass.

4.5. Conclusion: Overall, the results of this project indicate that 1) multiple viruses are present within ryegrass, 2) that ryegrass yield decreases overtime, 3) that viral load is higher in older ryegrass, and 4) that there is a potential link between lower yield and persistence and high/increasing levels of viral load. Whilst this research demonstrates that a tenable link between viruses and ryegrass yield deficit could exist, a definitive correlation between these two factors was not established during this research. Consequently, more research is necessary to elucidate the true impact of viruses upon ryegrass yield and persistence. However, prior to repeating such research, a range of improvements are required. For example, this research should be conducted for a longer period of time (10 months +), and ryegrass yield and performance should be compared across a range of locations using isogenic lines to eliminate the impact of genotypic variation. Furthermore, better environmental and/or cultural controls are necessary to minimize the effect of environmental variation. Furthermore, one may consider comparing the yield of virus free old and new ryegrass (grown in controlled environment) to the yield of virus infected ryegrass, to evaluate the extent of yield losses potentially attributable to viruses, as opposed to other factors. Essentially, before research of this nature is conducted again, it would be immensely beneficial to minimize and/or eliminate as many potential sources of variation and/or confounding factors as possible. Conducting research of this nature in a controlled manner could enable one to delineate the impact of viruses upon ryegrass yield and performance. Lastly, if one wishes to clearly ascertain or quantify the effect of mixed infection, one would need to compare the load and impact of single, as well as mixed or combined infections. This would also enable one to determine the nature of interactions between viruses. Whilst it remains impossible to entirely eliminate all potential sources of variation, mitigating the impact of as many variables as possible would enable one to establish the true impact of viruses. However, it remains plausible that viruses and/or age-related factors contribute to yield decline of pastures over time.

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Appendices:

Appendix 1: Yield Over Time

Date of cuts	Plot #	Line	Yield (grams)
24/04/15	1	LP256	11.6
	2	R164	12.9
	4	LP258	8.7
	6	BEALEY	10.3
	9	BEALEY	8.4
	11	R141	8.7
	15	LP258	8.1
	17	BEALEY	9.3
	19	LP258	8.1
	21	LP256	9.3
	23	R164	9.9
	24	R141	10.4
	27	R141	10.2
	29	LP256	8.3
	30	R164	8.3
18/05/15	1	LP256	13.3
	2	R164	12.8
	4	LP258	8.3
	6	BEALEY	11
	9	BEALEY	8.2
	11	R141	8.5
	15	LP258	7.8
	17	BEALEY	8.1
	19	LP258	7.8
	21	LP256	8
	23	R164	9.4
	24	R141	10.9
	27	R141	9.3
	29	LP256	8.4
	30	R164	8.6
15/06/15	1	LP256	9.8
	2	R164	9
	4	LP258	6.9
	6	BEALEY	8.3
	9	BEALEY	6.6
	11	R141	6.6
	15	LP258	6.8
	17	BEALEY	7.1

	19	LP258	7.1
	21	LP256	7.2
	23	R164	7.4
	24	R141	8.4
	27	R141	7.6
	29	LP256	7.1
	30	R164	7.3
9/12/15	2	R164	6.8
	4	LP258	11.5
	6	BEALEY	10.4
	9	BEALEY	9.1
	11	R141	13.5
	15	LP258	6.7
	17	BEALEY	9.7
	19	LP258	8.5
	21	LP256	8.1
	23	R164	9.4
	24	R141	10.9
	27	R141	10.4
	29	LP256	9.2
	30	R164	9.8
	1	LP256	7.5
23/12/15	2	R164	7.8
	4	LP258	9.4
	6	BEALEY	10
	9	BEALEY	8.9
	11	R141	10.3
	15	LP258	8.3
	17	BEALEY	7.9
	19	LP258	7.8
	21	LP256	7.4
	23	R164	8.6
	24	R141	10.5
	27	R141	7.7
	29	LP256	8.1
	30	R164	6.7
	1	LP256	7.9
11/01/16	2	R164	7.2
	4	LP258	8.1
	6	BEALEY	8.9
	9	BEALEY	7.4
	11	R141	7.7

	15	LP258	7.2
	17	BEALEY	7.1
	19	LP258	6.2
	21	LP256	7.8
	23	R164	7.9
	24	R141	7.6
	27	R141	7
	29	LP256	7.2
	30	R164	6
	3	R141	7.1
24/04/15	5	LP256	7.8
	7	R141	7.4
	8	LP256	7.6
	10	R164	6.9
	12	LP258	7.2
	13	LP258	8.7
	14	R141	8.3
	16	BEALEY	6.9
	18	R164	7.6
	20	BEALEY	7
	22	LP258	8.9
	25	LP256	9.9
	26	R164	7.6
	28	BEALEY	7
	3	R141	7
18/05/15	5	LP256	7.3
	7	R141	7.3
	8	LP256	6.9
	10	R164	6.7
	12	LP258	7.4
	13	LP258	8.8
	14	R141	7.1
	16	BEALEY	6.9
	18	R164	7.4
	20	BEALEY	6.9
	22	LP258	8.1
	25	LP256	7.1
	26	R164	7.2
	28	BEALEY	6.8
	3	R141	6.2
15/06/15	5	LP256	6.4
	7	R141	6.8

	8	LP256	6.5
	10	R164	6.3
	12	LP258	6.5
	13	LP258	6.9
	14	R141	6.4
	16	BEALEY	6.4
	18	R164	6.7
	20	BEALEY	6.6
	22	LP258	6.9
	25	LP256	7.2
	26	R164	6.5
	28	BEALEY	6.4
	3	R141	6.9
9/12/15	5	LP256	9.3
	7	R141	7.2
	8	LP256	7.7
	10	R164	8.1
	12	LP258	9.6
	13	LP258	10
	14	R141	5.9
	16	BEALEY	6.1
	18	R164	7.5
	20	BEALEY	7.1
	22	LP258	7.9
	25	LP256	5.8
	26	R164	7.3
	28	BEALEY	6.7
	3	R141	6.5
23/12/15	5	LP256	6.7
	7	R141	6.2
	8	LP256	6
	10	R164	6.9
	12	LP258	9.3
	13	LP258	5.7
	14	R141	5.6
	16	BEALEY	7.4
	18	R164	6.1
	20	BEALEY	7.9
	22	LP258	6.7
	25	LP256	6.4
	26	R164	6.2
	28	BEALEY	6.1

	3	R141	5.9
11/01/16	5	LP256	6
	7	R141	4.3
	8	LP256	5.7
	10	R164	6
	12	LP258	7.3
	13	LP258	4.7
	14	R141	5.3
	16	BEALEY	6
	18	R164	5.8
	20	BEALEY	6
	22	LP258	5.9
	25	LP256	6
	26	R164	5.8
	28	BEALEY	6.2

Turf Grass Yield Overtime

Plots	**18/02/16**	**24/03/16**	**5/05/16**
A	45.12	25.1	40.3
B	44.21	23.91	41.8
C	47.09	27.9	40.4
D	49.1	28.11	42.1
E	46.26	24.91	39.7

Appendix 2: ANOVA analysis of Yield

Field Trial Data:

Plots	18/02/16	24/03/16	5/05/16
A	45.12	25.1	40.3
B	44.21	23.91	41.8
C	47.09	27.9	40.4
D	49.1	28.11	42.1
E	46.26	24.91	39.7

Field Trial General ANOVA analysis:

Analysis of variance

Variate: Yield

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Date stratum	2	1110.631	555.316	400.33	
Date.*Units* stratum					
Plots	4	21.833	5.458	3.93	0.047
Residual	8	11.097	1.387		
Total	14	1143.561			

Message: the following units have large residuals.

Date 05-May-16
units 2 2.03 s.e. 0.86

Table of means	**Plots**	**A**	**B**	**C**	**D**	**E**
	Yield	36.84	36.64	38.46	39.77	36.96

Standard errors of differences of means

Table	Plots
rep.	3
d.f.	8
s.e.d.	0.962

Least significant differences of means (5% level)

Table	Plots
rep.	3
d.f.	8
l.s.d.	2.218

Fisher's unprotected least significant difference test

Plots

	Mean	
B	36.64	a
A	36.84	a
E	36.96	a
C	38.46	ab

D	39.77	b

General ANOVA for Yield Analysis (Old and New Ryegrass).

Analysis of variance

Variate: Yield

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	1.786	0.447	0.21	0.934
Old_new	1	115.841	115.841	53.95	<.001
Cultivar.Old_new	4	28.615	7.154	3.33	0.012
Residual	170	365.038	2.147		
Total	179	511.28			

All terms orthogonal, none aliased.

Message: the following units have large residuals.

units 4				5.18	s.e. 1.42
units 12	-8.12	s.e.	1.42		
units 46	4.27	s.e.	1.42		
units 55	4.24	s.e.	1.42		
units 58	4.14	s.e.	1.42		

Tables of means

Old_new	New	Old
	8.54	6.93

Cultivar	New	Old
Bealey	8.71	6.72
LP256	8.12	7.02
LP258	7.96	7.58
R141	9.23	6.52
R164	8.66	6.81

Standard errors of means

Table	Cultivar	Old_new	Cultivar Old_new
rep.	36	90	18
d.f.	170	170	170
e.s.e.	0.244	0.154	0.345

Standard errors of differences of means

Table	Cultivar	Old_new	Cultivar Old_new
rep.	36	90	18

d.f.	170	170	170
s.e.d.	0.345	0.218	0.488

Least significant differences of means (5% level)

Table	Cultivar	Old_new	Cultivar Old_new
rep.	36	90	18
d.f.	170	170	170
l.s.d.	0.682	0.431	0.964